(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
15 March 2001 (15.03.2001)

PCT

(10) International Publication Number
WO 01/17560 A1

(51) International Patent Classification[7]: A61K 39/395, 39/02, 39/085, 39/108, C12Q 1/00, 1/18, C07K 1/00, 16/00

(21) International Application Number: PCT/US00/24839

(22) International Filing Date:
11 September 2000 (11.09.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/153,310 10 September 1999 (10.09.1999) US

(71) Applicant *(for all designated States except US)*: CHILDREN'S MEDICAL CENTER CORPORATION [US/US]; 300 Longwood Avenue, Boston, MA 02115 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: BERNFIELD, Merton [US/US]; 25 Brimmer Street, Boston, MA 02108-1040 (US). PARK, Pyong, Woo [KR/US]; 1277 Commonwealth Avenue, Allston, MA 02134 (US).

(74) Agents: RESNICK, David, S. et al.; Nixon Peabody LLP, 101 Federal Street, Boston, MA 02110 (US).

(81) Designated States *(national)*: AU, CA, JP, US.

(84) Designated States *(regional)*: European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published:

— *With international search report.*

— *Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/17560 A1

(54) Title: METHOD FOR TREATING AND PREVENTING BACTERIAL INFECTION

(57) Abstract: The present invention provides methods and compositions for treating bacterial infections, especially infections by opportunistic pathogens in a subject by administering a compound that inhibits syndecan-1 shedding. The invention is based on the discovery that two diverse opportunistic pathogens, *S. aureus* and *P. aeruginosa*, enhance syndecan-1 shedding and that this shedding is critical for *Pseudomonas* pathogenesis via the respitory tract. The discovery is also based on the surprising finding that inhibition of syndecan-1 shedding prevents *Pseudomonas* pneumonia in a mammalian model. The *P. aeruginosa* shedding enhancer has been purified and identified as the manure 20 kDa LasA protein, a known virulence factor of *P. aeruginosa*.

9/prts

METHOD FOR TREATING AND PREVENTING BACTERIAL INFECTION

BACKGROUND OF THE INVENTION

This invention was made with government support under Grant No. R01 CA28735-15 by the National Institute of Health. The government has certain rights in the invention.

1. Field of the Invention

The present invention relates to methods and compositions for treating bacterial infections, especially infections by opportunistic pathogens.

2. Background

Microbial pathogenicity has been defined as the structural and biochemical mechanism whereby microorganisms cause disease. The first stage of microbial infection is the establishment of the pathogen at the portal of entry. Pathogens usually colonize host tissues that are in contact with the external environment. for example the urogenital tract, the digestive tract, the respiratory tract and the conjunctiva. Organisms that infect these regions have usually developed tissue adherence mechanisms and some ability to overcome or withstand host defense mechanisms usually resulting in colonization, multiplication, invasion or persistence of a pathogen on or within a host. Therefore, pathogenicity in bacteria may be associated with unique structural components of the cells, for example: capsules, fimbriae, lipolysaccharide (LPS) or other cell wall components; or, mechanisms such as active secretion of substances that either damage host tissues or protect the bacteria against host defenses. In most cases, a break in the host's first line defenses for example trauma, surgery, serious burns or indwelling devices can give rise to opportunistic infections. Examples of such pathogens are *Staphylococcus aureus*, hereafter referred to as *S. aureus*, and *Pseudomonas aeruginosa*, hereafter referred to as *P. aeruginosa*.

Human staphylococcal infections are frequent, but usually remain localized at the portal of entry by the normal host defenses. The portal may be a hair follicle, but

usually it is a break in the skin, which may be a minute needle stick or a surgical wound. Foreign bodies, including sutures, are readily colonized by staphylococci, which makes infection difficult to control (70). Another portal of entry is the respiratory tract. Staphylococcal pneumonia is a frequent complication of influenza (71). Serious consequences of staphylococcal infections occur when the bacteria invade the blood stream. A resulting septicemia may be rapidly fatal: a bacteremia may result in seeding other internal abscesses, other skin lesions, or infections of the lung, kidney, heart skeletal muscles or meninges. Staphylococcal disease has been a perennial problem in the hospital environment since the beginning of the antibiotic era. During the 1950's and early 1960's, staphylococcal infection was synonymous with nosocomial infection. Gram-negative bacilli for example, *Escherichia coli* and *P. aeruginosa* have replaced the staphylococci as the most frequent causes of nosocomial infections, although the staphylococci have remained a problem (70, 71).

P. aeruginosa is a major causative agent of gram-negative bacterial lung infections among compromised patients. Immunocompromised patients, such as neutropenic cancer and bone transplantation patients, are particularly susceptible to opportunistic infections (72). In this group of patients, *P. aeruginosa* is responsible for pneumonia and septicemia with attributable deaths reaching 30% (73, 74). *P. aeruginosa* is also one of the most common and lethal pathogens responsible for ventilator-associated pneumonia in intubated patients, with directly attributable death rates reaching 38% (75, 96). In nosocomial infections, *P. aeruginosa* is responsible for 16% of nosocomial pneumonia cases (70), 12% of hospital-acquired urinary tract infections (76), 8% of surgical wound infections (77), and 10% of bloodstream infections (9). In the expanding HIV-infected population, *P. aeruginosa* bacteremia is associated with 50% of deaths (79). In addition, *P. aeruginosa* corneal infection is one of the leading causes of control lens-related infection/loss of vision. *P. aeruginosa* also has a major impact on the rates of illness and death of patients with cystic fibrosis (80). Recent studies have also shown that *P. aeruginosa* associated pneumonia may play a role in many other lung manifestations, such as emphysema and chronic obstructive pulmonary disease. Despite the clinical data demonstrating the significance of *P. aeruginosa* in a range of infections, particularly lung infections, its pathogenic mechanism is not understood. The capacity of *P. aeruginosa* to

produce such diverse, often overwhelming infections is due to an arsenal of virulence factors.

Many extracellular virulence factors secreted by *P. aeruginosa* have been shown to be controlled by a complex regulatory circuit involving cell-to-cell signaling systems that allow the bacteria to produce these factors in a coordinated, cell-density-dependent manner (72). With respect to cystic fibrosis, hereafter referred to as C.F., the mechanisms contributing to pathogenesis are still largely unknown. In patients with C.F. the major clinical manifestations occur in the gastrointestinal tract and in the airways and lungs (81). The gastrointestinal tract symptoms of C.F. have been adequately managed in the last 30 to 40 years, but the major clinical problem facing these patients is the progressive loss of pulmonary function, usually due to chronic bacterial infections by mucoid *P. aeruginosa* - some strains of P. *aeruginosa* produce large amounts of extracellular polysaccharide and are referred to as mucoid strains (81). Once established as an infection in the lungs of C.F. individuals, *P. aeruginosa* can persist for decades. Although C.F. patients have intact immune systems they do not mount an effective response against *P. aeruginosa*. Ordinarily, opsonic antibodies are a crucial component of immunity to *P. aeruginosa*, but during such chronic infections, *P. aeruginosa* produces MEP/alginate - a factor central to microbial persistence - as it is responsible for eliciting antibodies that fail to mediate phagocytosis of *P. aeruginosa*. Moreover, the eradication of colonization with *P. aeruginosa* is almost impossible in C.F. patients because of the emergence of multidrug-resistant strains and the protective effects of alginate (81).

The increasing emergence of bacterial strains resistant to antibacterial drugs has been driven mainly by overuse of antibacterial agents, thereby selecting for bacteria resistant to these agents (82). Beginning with the use of penicillin in the 1940's, drug resistance developed in staphylococci within a very short time after introduction of an antibiotic into clinical use. *S. aureus* responded to the introduction of antibiotics by the usual bacterial means to develop resistance, for example by mutation in chromosomal genes followed by selection of resistant strains, and by acquisition of resistance genes as extra chromosomal plasmids, transducing particles, transposons, or other types of DNA inserts. *S. aureus* expresses its resistance to drugs and antibiotics by a variety of mechanisms (83, 84, 85, 86). Hospital strains of *S.*

aureus are commonly resistant to a variety of different antibiotics. Methicillin resistance has been widespread since the late 1960s, and some *S. aureus* strains are now resistant to all clinically useful antibiotics except the glycopeptides vancomycin and teicoplanin (87). In the last several years, vancomycin-resistant *S. aureus* strains have emerged, sparking fears of an untreatable strain of this important nosocomial pathogen (88,89,90,91). In addition, *S. aureus* expresses resistance to antiseptics and disinfectants, such as quaternary ammonium compounds, which may aid in its survival in the hospital environment (92). Another example is *P. aeruginosa* which has become one of the most problematic human pathogens, as it shows intrinsic resistance to many structurally unrelated antibiotics. Resistance mechanisms include low outer membrane permeability or multidrug efflux pumps (tetracycline, imipenem, fluoroquinolones, aminoglycosides) or production of antibiotic modifying enzymes (aminoglycosides, β-lactams) (94). Furthermore, previous exposure to antibiotics often leads to multidrug-resistant *P. aeruginosa* strains (95). The pathogenesis of *P. aeruginosa* and *S. aureus* is clearly mutifactorial as underlined by the large number of virulence factors and the broad spectrum of diseases these bactera cause. It is, therefore, imperative to elucidate the mechanisms of bacterial pathogenesis, for example *S. aureus* and *P. aeruginosa*, and to develop alternative prophylactic and therapeutic agents that target specific host-pathogen interactions involved in bacterial pathogenesis.

Shedding is a process in which cell surface proteins are cleaved by proteinases known collectively as sheddases or secretases, and released from the surface as soluble effectors (1-3). It is an important biological mechanism of protein secretion and activation for the approximately 1% of total cell surface proteins that can also function as soluble ectodomains. Numerous types of surface molecules are shed as soluble ectodomains and the list includes cytokines, growth factors and their receptors, enzymes and cell adhesion molecules such as selectins (4), CD14 (5), EGF (6), TNF-α (7,8) and their receptors (9,10), IL-6 receptor (11), Fas ligand (12) and TGF-α (13), to name a few. It is well established that these shed ectodomains play pivotal roles in diverse pathophysiological events including septic shock, host defense and wound healing. Furthermore, shedding provides an additional level of regulating the activity of affected effectors since shedding itself has been found to be regulated

by various extracellular ligands (14-16) and intracellular signaling pathways (3,13,16,17).

Pathogenic microorganisms, constantly evolving in the hostile host environment, have learned to frequently take advantage of existing host systems for their pathogenesis. For example, a diverse group of pathogens including *Yersinia* spp. (20), *Bordetella pertussis* (21,22) and adenovirus (23) express RGD-containing cell surface ligands and use these "molecular mimics" to interact with host integrin receptors for their colonization (24). Bacteria also contain molecules in their pathogenic arsenal that can derange host homeostasis to their benefit. Several bacteria secrete toxins that can modify the host cell cytoskeleton (25) and secrete enzymes that can degrade extracellular matrix (ECM) components, immunoglobulins and complement, either directly (26,27) or indirectly by activating the host's matrix metalloproteinases (28). Furthermore, LPS from Gram negative bacteria, the causative agent of endotoxic shock, affects expression of many host defense effectors such as TNF-α and IL-1, -6, -8 and -10 (29).

Recent studies indicate that bacterial pathogens may also utilize the host's shedding mechanism to the benefit of their pathogenesis. For instance, the pore-forming toxins, streptolysin O and *E. coli* hemolysin, have been found to trigger shedding of LPS (CD14) and IL-6 receptors (30). Culture supernatants from *P. aeruginosa*, *S. aureus*, *Serratia marcescens* and *Listeria monocytogenes* can also augment shedding of the IL-6 receptor (31), and culture supernatants from *Staphylococcus epidermidis* can activate shedding of TNF-α (32), although the responsible shedding enhancers have not been defined in these studies. Furthermore, increased serum levels of soluble ectodomains of several surface effectors, such as CD14, TNF-α and IL-4 receptors, have been documented during infection (33-35). These findings suggest that bacteria-enhanced shedding can modulate the activation and function of host effectors, and play a role in bacterial pathogenesis.

Syndecans are a family of cell surface heparan sulfate proteoglycans (HSPGs) which, along with the glypicans, are the major source of cell surface heparan sulfate (HS) (36). There are currently four mammalian syndecans, syndecan-1 through -4, each encoded by distinct genes. Functionally, syndecans can bind and modulate the activity of a diverse group of soluble and insoluble ligands, such as ECM

components, growth factors, chemokines, cytokines and proteases, through the action of their HS chains. Syndecans have also been proposed to act as adhesion and internalization receptors for pathogenic microorganisms (37,38). The extracellular domains of syndecans can be shed as soluble, intact HSPGs which, because they bind the same ligands as their precursor proteoglycans on the cell surface, can serve as soluble effectors. For example, shed syndecan-1 ectodomains have been found to regulate the proliferative response of cells to FGF-2 (39) and potentiate the activity of neutrophil enzymes, such as elastase and cathepsin G (40), by binding to the enzymes and protecting them from inhibition by their physiological inhibitors. All syndecans are shed constitutively in culture, but available evidence also indicates that syndecan shedding is highly regulated and is a host response to tissue injury (16). There has been no reported association between syndecan-1 shedding and bacterial pathogenesis.

There continues to exist a need in the art for new antimicrobial methods and materials, especially against opportunistic pathogens.

SUMMARY OF THE INVENTION

The present invention provides methods and compositions for treating bacterial infections, especially infections by opportunistic pathogens in a subject by administering a compound that inhibits syndecan-1 shedding. The invention is based on the discovery that two diverse opportunistic pathogens, *S. aureus* and *P. aeruginosa*, enhance syndecan-1 shedding and that this shedding is critical for *Pseudomonas* and staphylococcal pathogenesis via the respiratory tract. The discovery is also based on the surprising finding that inhibition of syndecan-1 shedding prevents *Pseudomonas* pneumonia in a mammalian model. The *P. aeruginosa* shedding enhancer has been purified and identified as the mature 20 kDa LasA protein, a known virulence factor of *P. aeruginosa*.

According to one aspect of the invention a method is provided for treating a bacterial infection comprising:

a) determining whether the bacterium causing the infection is capable of enhancing host cell syndecan-1 shedding; and

b) administering an effective amount of a compound that inhibits syndecan-1 shedding to a subject suffering from said infection, wherein the compound is selected from (a) inhibitors of a bacterial factor responsible for syndecan-1 cleavage, e.g., LasA in the case of *P. aeruginosa,* (b) compounds that bind the syndecan and disrupt cleavage, (c) syndecan-1 decoys, and (d) inhibitors of the host cell shedding mechanism, except when the bacterium is *Pseudomonas aeruginosa*, the compound is not genistein or tyrphostin A47. More preferably, when the bacterium is *Pseudomonas aeruginosa*, the compound is not a general protein tyrosine kinase inhibitor.

Inhibitors of the host cell shedding mechanism include hydroxamate derivatives and protein tyrosine kinase inhibitors. BB1101 is a preferred hydroxamate derivative. Genistein, herbinycen and tyrphostins, such as A25, are preferred protein tyrosine kinase inhibitors.

Compounds that bind the syndecan and disrupt cleavage include polyclonal and monoclonal antibodies and peptides.

The method of the present invention is useful in treating infections of the respiratory system, the urinary tract, the skin, and blood stream. The method is particularly useful in treating *Pseudomonas* or *Staphylococcus* lung infections.

In other embodiments the invention provides methods for screening compounds that inhibit the syndecan-1 shedding. The methods comprise contacting cells which express syndecan-1, such as NMuMG cells, with the compound being screened and measuring syndecan-1 cleavage.

The invention also provides a method for diagnosing an infection by a bacterial organism that is capable of being treated by the methods of the present invention. The method comprises testing a bacterial sample for the ability to induce syndecan-1 cleavage.

Other aspects of the invention are disclosed *infra*.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Culture supernatants of *P. aeruginosa* and *S. aureus* enhance shedding of syndecan-1 by NMuMG cells. Bacteria were grown overnight in TSB at 37°C to stationary growth phase and culture supernatants were collected. Fresh

NMuMG culture media (media control) or filter-sterilized bacterial supernatants diluted to 20% (v/v) with NMuMG media were incubated with confluent NMuMG cells in 96 well plates for 14 h at 37°C. At the end of incubation, conditioned media were collected, centrifuged to remove cells, acidified and dot blotted onto cationic Immobilon N PVDF membranes. Extent of syndecan-1 shedding enhancement was determined by the dot immunoblotting method as described in "Experimental Procedures". Each data point represents mean of duplicate or triplicate measurements, and results are presented as fold over media control. The number and horizontal bar in *P. aeruginosa* and *S. aureus* samples indicate mean values for these bacteria.

Figure 2. Concentration- and time-dependent enhancement of syndecan-1 shedding by *P. aeruginosa* supernatant. Confluent NMuMG cells in 96 well plates were incubated with A) varying concentrations (0.1-20%, v/v) of filter-sterilized *P. aeruginosa*, strain BL2, supernatant for 20 h at 37°C or B) 20 % (v/v) BL2 supernatant for 2, 8, or 20 h at 37°C. Cell surface and shed syndecan-1 levels were quantified by the dot immunoblotting method as described previously. Measurements obtained with fresh NMuMG media served as control values. Error bars represent SD determined from triplicate measurements.

Figure 3. *P. aeruginosa* supernatant enhances shedding of syndecan-1 by various host cells. Filter sterilized supernatant (20%, v/v) of the clinical *P. aeruginosa* isolate, BL2, was incubated with 1 day post-confluent NMuMG (96 well), C127 (96 well) and LA-4 (24 well) epithelia, and NIH3T3 fibroblasts (24 well) for 20 h at 37°C. Syndecan-1 shed under these conditions was quantified as described previously. Results are presented as mean fold increase over media control±SD.

Figure 4. The syndecan-1 shedding enhancer of *P. aeruginosa* is a 10-30 kDa protein. Overnight culture supernatants of strain BL2 were treated with 10 μg/ml proteinase K for 30 min at 37°C, inactivated with 20 mM PMSF, spun down to remove precipitates and filter sterilized. Alternatively, culture supernatants were centrifuged in MWCO spin microfuge tubes at 10,000g for 40 min and flow through fractions were collected. The retentate materials were collected by resuspending the semi-dried material above the MWCO membrane with fresh TSB. These samples, along with untreated BL2 supernatant, were filter sterilized, diluted to 20% (v/v) with

NMuMG media and incubated with confluent cultures of NMuMG cells in 96 well plates for 14 h at 37°C. Shed syndecan-1 levels were quantified by the dot immunoblotting method as described previously. Error bars represent SD determined from triplicate measurements.

Figure 5. Partial purification of *P. aeruginosa* supernatant by ammonium sulfate precipitation and gel chromatography identifies a single 20 kDa protein as a candidate syndecan-1 shedding enhancer. An overnight culture supernatant of strain BL2 was precipitated by 80% ammonium sulfate and fractionated by Bio-Gel P-30 gel chromatography at a flow rate of 4.5 ml/h. The collected fractions were speed-vac dried, resuspended in NMuMG culture media, filter sterilized and assayed for syndecan-1 shedding activity. Shed syndecan-1 levels were quantified by the dot immunoblotting method as described previously. Each data point in the activity chromatogram represents mean values from duplicate determinations. The active fractions (12 & 13) and inactive fractions in the vicinity (10, 11, 13, 14 & 15) were subjected to 12% reducing SDS-PAGE and visualized by Coomassie Brilliant Blue R-250 staining (insert).

Figure 6. Immunoaffinity chromatography with anti-LasA IgGs demonstrates that the *P. aeruginosa* shedding enhancer is LasA. The partially purified material obtained from ammonium sulfate precipitation and gel chromatography of *P. aeruginosa* supernatant was fractionated by mouse polyclonal anti-LasA IgG affinity chromatography. The flow through (FT), wash (WSH) and eluate (EL) fractions were collected, and along with the starting material (fractions 12&13), tested for their ability to enhance shedding of syndecan-1 by NMuMG cells as previously described. Results of the activity assay are presented as mean fold increase over media control±SD. Results from analysis of the fractions by 12% SDS-PAGE and Coomassie staining are shown in the insert.

Figure 7. Purified LasA enhances shedding of syndecan-1 by various host cells in a concentration-dependent manner. Varying concentrations of LasA (0.5, 5, 10 μg/ml), purified by consecutive steps of ammonium sulfate precipitation, gel chromatography and immunoaffinity chromatography, were incubated with confluent cultures of NMuMG (96 well), LA-4 (24 well), or NIH3T3 (24 well) cells for 8 h at

37°C. Extent of syndecan-1 shedding enhancement was determined as previously described. Error bars represent SD determined from triplicate measurements.

Figure 8. Syndecan-1 ectodomains induced to shed by purified LasA and crude *P. aeruginosa* supernatant are intact proteoglycans and the size of their core proteins are identical to that of the constitutively shed ectodomain. Conditioned media from unstimulated NMuMG cells (lanes 1 & 4) and from NMuMG cells stimulated with 5 μg/ml purified LasA (lanes 2 & 5) or 20% (v/v) crude *P. aeruginosa* supernatant (lanes 3 & 6) were incubated with DEAE-Sephacel for 2 h at 4°C, and bound materials were eluted with 2 M NaCl. Approximately 30 ng of the undigested samples were analyzed by 3.5-10% gradient SDS-PAGE and Western immunoblotting using the 281-2 anti-syndecan-1 monoclonal antibody (undigested, lanes 1-3) or digested with 10 mU/ml heparitinase and 20 mU/ml chondroitin sulfate ABC lyase and then analyzed by SDS-PAGE and Western immunoblotting (digested, lanes 4-6). Molecular masses of the immunoreactive proteins were approximated from the migration pattern of pre-stained size standards.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides methods and compositions to treat and prevent bacterial infections. As one aspect of the present invention, it has now been discovered that shedding of syndecan-1 plays a role in infection by opportunistic pathogens such as *P. aeruginosa* and *S. aureus* and that inhibiting syndecan-1 shedding can be used to treat infection by such opportunistic pathogens.

As used herein, the terms "treatment" or "treating" include: (1) preventing such disease from occurring in a subject who may be predisposed to these diseases but who has not yet been diagnosed as having them; (2) inhibiting these diseases, i.e., arresting their development; or (3) ameliorating or relieving the symptoms of these diseases, i.e., causing regression of the disease states.

Compounds which can inhibit sydecan shedding are selected from (a) inhibitors of a bacterial shedding enhancer responsible for syndecan-1 cleavage, (b) compounds that bind the syndecan and disrupt cleavage, (c) syndecan-1 decoys, and (d) inhibitors of the host cell shedding mechanis. The compounds preferably inhibit syndecan-1 shedding by at least 50% in the syndecan shedding assay of Subramanian,

et al., *J. Biol. Chem.* 272. 14713-14720 (1997). More preferably the compounds inhibit syndecan-1 shedding by 75%, most preferably 95%. Additional compounds are identified and tested in the screening assays discussed in more detail below.

Determining whether the bacterium causing the infection is capable of enhancing host cell syndecan-1 shedding can be done prior to treatment by using a host cell (e.g., epithelial cells) containing sample bacterium suspected of causing the infection to see if syndecan-1 shedding is occurring. Alternatively, a determination that a particular species of bacteria enhances syndecan-1 shedding can be made at an earlier date and one needs merely determine that the species being treated is one known to enhance such shedding.

The invention provides efficient screening methods to identify pharmacological agents or lead compounds for agents which inhibit syndecan-1 shedding. The methods are amenable to automated, cost-effective high throughput drug screening and have immediate application in a broad range of pharmaceutical drug development programs.

A preferred assay mixture of the invention comprises a cell expressing syndecan-1, for example a normal murine mammary gland (NMuMG) cell. An assay mixture of the invention also comprises a candidate pharmacological agent. Generally a plurality of assay mixtures are run in parallel with different candidate agent concentrations to obtain a differential response to the various concentrations. Typically, one of these assay mixtures serves as a negative control, i.e. at zero concentration or below the limits of assay detection. Candidate agents encompass numerous chemical classes, though typically they are organic compounds and preferably small organic compounds. Small organic compounds suitably may have e.g. a molecular weight of more than about 50 yet less than about 2,500. Candidate agents may comprise functional chemical groups that interact with proteins and/or DNA.

Candidate agents are obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides and peptides.

Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced.

Additionally, natural and synthetically produced libraries and compounds are readily modified through conventional chemical, physical, and biochemical means. In addition, known pharmacological agents may be subject to directed or random chemical modifications, such as acylation, alkylation, esterification, amidification, etc.

The resultant mixture is incubated under conditions whereby, but for the presence of the candidate pharmacological agent, the syndecan-1 would be cleaved from the cell surface. Incubations may be performed at any temperature which allows syndecan shedding, typically between 25°C and 40°C. Incubation periods are typically between 0.1 and 10 hours, preferably less than 5 hours, more preferably less than 2 hours.

After incubation, the presence or absence of the shed syndecan is detected by any convenient method, including ELISA, radioimmunoassay, or dot immunoblotting of cell-free supernatant.

Particularly useful inhibitors of the host cell syndecan-1 shedding mechanism, and thus effective inhibitors of bacterial infection are hydroxamate derivatives, e.g., BB1101 (British BioTech), and protein tyrosine kinase (PTK) inhibitors, e.g., genistein, herbimycin A and tyrphostin A25.

Preferred protein tyrosine kinase inhibitors are inhibitors of the Src family of protein kinases.

Syndecan-1 decoys can also be used to inhibit syndecan-1 shedding. Syndecan-1 decoys include synthetic peptides (linear or cyclized); recombinant peptides corresponding to the juxtamembrane domain of syndecan-1; shorter peptides corresponding to the cleavage domain of syndecan-1, usually 10-15 amino acids in length; organic compounds, similar in structure to the juxtamembrane region or cleavage cite peptide sequence; and anti-idiotypic antibodies directed against the Fab domain of the antibody recognizing the cleavage site sequence.

Antibodies and binding fragments thereof that bind syndecan-1 and prevent cleavage are also useful for inhibition.

The antibodies and binding fragments thereof can be either polyclonal or monoclonal, but preferably are monoclonal. If polyclonal, they can be in the form of antiserum or monospecific antibodies, such as purified antiserum which has been produced by immunizing animals with purified syndecan-1. Preferably, however, the antibodies are monoclonal antibodies so as to minimize the administration of extraneous proteins to an individual. Monoclonal antibodies can be prepared according to well known protocols. See. e.g., Skare et al., *J. Biol. Chem.* 268: 16302-16308 (1993), U.S. Pat. Nos. 4,918,163 and 5,057,598, which are incorporated herein by reference. The antibodies can be whole, Fab's, single chain, single domain heavy chain, etc. Single chain antibodies are preferable. Methods for the production of single chain binding polypeptides are described in detail in, e.g., U.S. Pat. No. 4,946,778, which is incorporated herein by reference.

For administration to humans, e.g., as a component of a composition for *in vivo* treatment, the monoclonal antibodies are preferably substantially human to minimize immunogenicity, and are in substantially pure form. By "substantially human" is meant that the immunoglobulin portion of the composition generally contains at least about 70% human antibody sequence, preferably at least about 80% human, and most preferably at least about 90-95% or more of a human antibody sequence.

For therapeutic applications, the compounds may be suitably administered to a subject such as a mammal, particularly a human, alone or as part of a pharmaceutical composition, comprising the compounds together with one or more acceptable carriers thereof and optionally other therapeutic ingredients. The carrier(s) must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipient thereof.

The pharmaceutical compositions of the invention include those suitable for oral, rectal, nasal, topical (including buccal and sublingual), vaginal, parenteral (including subcutaneous, intramuscular, intravenous and intradermal), occular using eye drops, transpulmonary using aerosolubilized or nebulized drug administration. Oral and nasal administration are preferred in the present case. The formulations may conveniently be presented in unit dosage form, e.g., tablets and sustained release capsules, and in liposomes, and may be prepared by any methods well know in the art

of pharmacy. See, for example, *Remington's Pharmaceutical Sciences*, Mack Publishing Company, Philadelphia, PA (17th ed. 1985).

Such preparative methods include the step of bringing into association with the molecule to be administered ingredients such as the carrier which constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers, liposomes or finely divided solid carriers or both, and then if necessary shaping the product.

Compositions of the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets or tablets each containing a predetermined amount of the active ingredient; as a powder or granules; as a solution or a suspension in an aqueous liquid or a non-aqueous liquid; or as an oil-in-water liquid emulsion or a water-in-oil liquid emulsion, or packed in liposomes and as a bolus, etc.

A tablet may be made by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine the active ingredient in a free-flowing form such as a powder or granules, optionally mixed with a binder, lubricant, inert diluent, preservative, surface-active or dispersing agent. Molded tablets may be made by molding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent. The tablets optionally may be coated or scored and may be formulated so as to provide slow or controlled release of the active ingredient therein.

Compositions suitable for topical administration include lozenges comprising the ingredients in a flavored basis, usually sucrose and acacia or tragacanth; and pastilles comprising the active ingredient in an inert basis such as gelatin and glycerin, or sucrose and acacia.

Compositions suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example, sealed ampules and vials, and may

be stored in a freeze dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets.

The compound can be used in a concurrent administration with one or more antibiotics. "Concurrent administration," or co-treatment, as used herein includes administration of the agents, in conjunction or combination, together, or before or after each other. The compound and antibiotics may be administered by different routes. For example, the compound may be administered intravenously while the antibiotics are administered intramuscularly, intravenously, subcutaneously, orally or intraperitoneally. Alternatively, the compound may be administered intraperitoneally while the antibiotics are administered intraperitoneally or intravenously, or the compound may be administered in an aerosolized or nebulized form while the antibiotics are administered, e.g., intravenously. The compound and antibiotics are preferably both administered intravenously. The compound and antibiotics may be given sequentially in the same intravenous line, after an intermediate flush, or may be given in different intravenous lines. The compound and antibiotics may be administered simultaneously or sequentially, as long as they are given in a manner sufficient to allow both agents to achieve effective concentrations at the site of infection.

Therapeutic effectiveness is based on a successful clinical outcome, and does not require that the antimicrobial agent or agents kill 100% of the organisms involved in the infection. Success depends on achieving a level of antibacterial activity at the site of infection that is sufficient to inhibit the bacteria in a manner that tips the balance in favor of the host. When host defenses are maximally effective, the antibacterial effect required may be minimal. Reducing organism load by even one log (a factor of 10) may permit the host's own defenses to control the infection. In addition, augmenting an early bactericidal/bacteriostatic effect can be more important than long-term bactericidal/bacteriostatic effect. These early events are a significant and critical part of therapeutic success, because they allow time for host defense mechanisms to activate.

It will be appreciated that actual preferred amounts of a given compound used in a given therapy will vary according to the particular compound being utilized, the particular compositions formulated, the mode of application, the particular site of administration, the patient's weight, general health, sex, etc., the particular indication being treated, etc. and other such factors that are recognized by those skilled in the art including the attendant physician or veterinarian. Optimal administration rates for a given protocol of administration can be readily determined by those skilled in the art using conventional dosage determination tests.

The present invention is further illustrated by the following Examples. These Examples are provided to aid in the understanding of the invention and are not construed as a limitation thereof.

EXAMPLE 1

Materials

Affi-Prep Hz Hydrazide affinity chromatography resins, Bio-Gel P-30 gel chromatography resins, Coomassie Brilliant Blue R-250 and pre-stained SDS-PAGE size standards were purchased from Bio-Rad (Melville, NY). Bisindolylmaleimide I, genistein and Tyrphostin A25 were from Calbiochem (La Jolla, CA). Heparan sulfate lyase (heparitinase) and chondroitin sulfate ABC lyase were obtained from Seikagaku (Ijamsville, MD). Tryptic soy broth (TSB) and tryptic soy agar were purchased from Remel (Lenexa, KS). The cationic PVDF membrane, Immobilon N, was from Millipore (Bedford, MA) and ProBlott PVDF membrane for N-terminal sequencing was from Applied Biosystems (Foster City, CA). Tissue culture media and supplements other than serum were from Mediatech (Herndon, VA), fetal calf and calf serum were from HyClone (Logan, UT) and tissue culture plastics were from Costar (Corning, NY). Enhanced chemiluminescense (ECL) Western blotting detection reagents were from Amersham (Arlington Heights, IL), DEAE Sephacel was from Pharmacia Biotech (Piscataway, NJ) and molecular weight cutoff spin tubes were from Pall Filtron (Northborough, MA). TPCK-treated trypsin, soybean trypsin inhibitor and all other materials were purchased from Sigma (St. Louis, MO).

Cells and immunochemicals

Normal murine mammary gland (NMuMG), mouse lung adenoma (LA-4) and mouse mammary gland (C127) epithelia, and Swiss mouse embryo fibroblasts (NIH3T3) were purchased from the ATCC (Rockville, MD). The cells were cultured as previously described (41) according to the provider's recommendations.

P. aeruginosa laboratory strains 7700 and 10145 were from the ATCC. The clinical *P. aeruginosa* isolates, BL1, BL2, CF1, CF2 and SP1, were from the Division of Infectious Diseases at Washington University School of Medicine (St. Louis, MO), CT4 was kindly provided by Dr. David Roberts at the NCI (Rockville, MD) (42) and PAO1 was maintained in the Pier lab. *S. aureus* laboratory strains 8095, 10832 (Woods), 12598 (Cowan) and 25904 (Newman) were from the ATCC. The clinical blood isolates of *S. aureus*, 070-0875, 093-0861, 108-0009, 111-0449 and 116-0031, were obtained from the Division of Infectious Diseases at Washington University School of Medicine. The *Salmonella enteritidis* clinical isolate was kindly provided by Dr. Robert Thompson of the Department of Vascular Surgery at Washington University School of Medicine. The laboratory strains of *Staphylococcus saprophyticus* (15305), *Staphylococcus xylosus* (29971), *Salmonella enteritidis* (10376), *Salmonella typhimurium* (14028), *Streptococcus pneumoniae* (27336) and *Klebsiella pneumoniae* (27736) were from the ATCC.

The rat monoclonal anti-mouse syndecan-1 ectodomain antibody (281-2) was generated in the Bernfield laboratory (43) and is now commercially available from Pharmingen (San Diego, CA). The mouse polyclonal anti-LasA antibody was generated in the Pier laboratory. Horse radish peroxidase-conjugated goat anti-rat secondary antibodies were obtained from either Jackson Immunoresearch (West Grove, PA) or Cappel (Durham, NC).

Syndecan shedding assays

Quantification of syndecan shedding was performed as previously described (16). Briefly, confluent or 1 day post-confluent cultures of NMuMG and C127 cells in 96 well plates and LA-4 and NIH3T3 cells in 24 well plates were washed once with their respective culture media, and various test samples diluted in culture media were added to the cells. Cells were incubated at 37°C with the samples for the indicated times as described in the figure legends. Cell viability was measured with the

tetrazolium salt (MTT) conversion assay (44). For quantification of shedding, the culture supernatants were collected, spun down to remove cells and the cell-free supernatants were applied to Immobilon N membranes using the dot immunoblotting apparatus. To obtain measurements within the linear range of the dot immunoblotting quantification method, 70 µl out of 100 µl in each 96 well were applied for NMuMG and C127 cells, and 200 µl out of 500 µl in each 24 well were applied for LA-4 and NIH3T3 cells. The samples were acidified by adding NaOAc (pH 4.5), NaCl and Tween-20 to final concentrations of 50 mM, 150 mM and 0.1% (v/v), respectively. By acidifying the samples, mostly highly anionic molecules such as proteoglycans are retained by the cationic PVDF membrane during dot blotting. For quantification of cell surface syndecan-1, following removal of media with or without test samples, cells were washed once with ice-cold TBS (50 mM Tris, pH 7.5, 150 mM NaCl) containing 0.5 mM EDTA and incubated for 15 min at 4°C with ice-cold 10 µg/ml TPCK-treated trypsin in TBS with 0.5 mM EDTA. Trypsin was subsequently inactivated with 100 µg/ml soybean trypsin inhibitor and the reaction mixture was spun down to remove detached cells. For NMuMG cells, 15 µl out of 100 µl trypsinate were blotted onto Immobilon N membranes as described above to obtain measurements in the linear range of the dot immunoblotting method. Development of the blotted membranes were carried out by incubations at 4°C with i) 10% (w/v) non-fat dry milk in TBS for 2 h or longer, ii) 0.2 µg/ml of anti-syndecan-1 antibody (281-2) in BLOTTO (TBS containing 0.5% non-fat dry milk and 0.1% Tween-20) for 14-24 h, iii) BLOTTO for 30 min x 2, iv) 1:8,000 dilution of horse radish peroxidase-conjugated goat anti-rat antibodies in BLOTTO for 14-24 h, v) TBS for 30 min x 2 and vi) the ECL development reagent. The developed blots were scanned and quantified using the public domain NIH Image (V. 1.60) software.

Ammonium sulfate precipitation and Bio-Gel P-30 gel chromatography

Overnight culture supernatant (1 L) of *P. aeruginosa*, strain BL2, was precipitated overnight at 4°C with ammonium sulfate at 80% saturation. The resulting precipitate was pelleted by centrifugation at 15,000 x g for 30 min at 4°C, dissolved in 60 ml de-ionized H_2O, and dialyzed twice against 4 L of de-ionized H_2O.

The dialysate was freeze-dried, resuspended in 30 ml buffer A (50 mM HEPES, pH 7.5, 50 mM NaCl), and 5 ml of this sample was applied to a 1 x 115 cm Bio-Gel P-30 column pre-equilibrated with buffer A. The applied material was fractionated at a flow rate of 4.5 ml/h with buffer A and twenty-four one hour fractions were collected. Aliquots of each fraction were speed-vac dried, resuspended in NMuMG culture media, filter sterilized and tested for their ability to enhance syndecan-1 shedding by NMuMG cells. For gel analysis, fractions were dialyzed against de-ionized water, speed-vac dried, resuspended in SDS-PAGE sample buffer and fractionated by 12% reducing SDS-PAGE.

Immunoaffinity chromatography

Carbohydrate moieties within the Fc region of anti-LasA IgGs were oxidized and reacted with hydrazide groups in the Affi-Prep coupling resin to form covalent hydrazone bonds. This coupling method was employed to orient the antigen binding sites outwards from the resin to potentially achieve higher antigen binding capacities. Mouse polyclonal anti-LasA IgGs were purified by protein G affinity chromatography and dialyzed into oxidation buffer (0.1 M NaOAc, pH 5.5, 1 M NaCl). Anti-LasA IgGs (2 mg) in 5 ml of oxidation buffer were oxidized by incubation for 1 h at room temperature in the dark with 500 µl of 180 mg/ml $NaIO_4$ in de-ionized H_2O. The oxidized antibody was first dialyzed into H_2O, then into coupling buffer (0.1 M NaOAc, pH 4.5, 1 M NaCl) and incubated overnight with 2 ml of Affi-Prep Hydrazide gel slurry at 4°C. The coupled affinity resin was transferred to a polypropylene column and the active fractions from gel chromatography, resuspended in binding buffer (50 mM HEPES, pH 7.5, 150 mM NaCl), were applied. The samples were re-cycled overnight at a flow rate of 5 ml/h through the affinity resin at 4°C. The flow through fraction was collected and the column was washed with binding buffer. The specifically bound materials were eluted with 0.1 M glycine (pH 2.8), neutralized by 0.1 M HEPES (pH 7.5), dialyzed into autoclaved de-ionized H_2O and concentrated by lyophilization. The concentration of purified LasA was determined by UV spectrophotometry based on the number of tyrosine and tryptophan residues in LasA (1_{A280}=0.41 mg/ml).

Western immunoblotting of partially purified syndecan-1 ectodomains

Conditioned media from unstimulated NMuMG cells (constitutively shed), and from NMuMG cells stimulated with crude *P. aeruginosa* supernatant (20%, v/v) or purified LasA (5 μg/ml) were collected, and NaOAc (pH 4.5) and NaCl were added to final concentrations of 100 and 300 mM, respectively. The acidified conditioned media were incubated with DEAE-Sephacel for 2 h at 4°C. The mixtures were applied to disposable polypropylene columns, washed with 100 mM NaOAc, pH 4.5, 300 mM NaCl buffer and bound materials were eluted with 2M NaCl. The eluates were dialyzed extensively against de-ionized H_2O, concentrated by lyophilization and the amount of partially purified syndecan-1 in the samples was estimated by dot immunoblotting. Approximately 30 ng from each sample was resuspended in digestion buffer (50 mM Tris, pH 7.5, 50 mM NaOAc, 5 mM EDTA, 2 mM PMSF) and digested with 10 mU/ml heparitinase and 20 mU/ml chondroitin sulfate ABC lyase for 3 h at 37°C with fresh enzymes added after 1.5 h. These digested samples and 30 ng of undigested samples were fractionated by SDS-PAGE using 3.5-10% gradient acrylamide gels, electrophoretically transferred to Immobilon N (undigested) or nitrocellulose (digested) membranes, probed with monoclonal rat anti-mouse syndecan-1 antibodies (281-2) and then horse radish peroxidase-conjugated goat anti-rat IgGs, and developed by the ECL detection method as described above.

RESULTS

P. aeruginosa and S. aureus secrete soluble enhancers of syndecan-1 shedding

To initiate our study examining the interactions between bacterial pathogens and the host's syndecan shedding system, we screened overnight culture supernatants from several Gram negative and positive bacteria for their ability to alter shedding of syndecan-1 by NMuMG cells. NMuMG cells were chosen initially because they express all syndecans, especially syndecan-1 and -4 abundantly (41), the shedding assays are routine in our laboratory with this epithelial cell line and also because the host epithelia is the target cell type of many bacterial pathogens (45,46). Overnight culture supernatants of bacteria were filter sterilized, diluted to 20% (v/v) with NMuMG culture media and incubated with NMuMG cells for 14 h at 37°C. As shown in figure 1, culture supernatants from all tested *P. aeruginosa* (7/7 clinical, 2/2

laboratory) and majority of *S. aureus* (3/5 clinical, 3/4 laboratory) strains enhanced shedding of syndecan-1 by more than 4-fold control levels, whereas strains from several other Gram negative (*Salmonella enteritidis, Salmonella typhimurium, Klebsiella pneumoniae*) and positive (*Staphylococcus saprophyticus, Staphylococcus xylosus, Streptococcus pneumoniae*) bacteria did not. Cellular extracts of *P. aeruginosa* and *S. aureus* strains did not affect shedding (data not shown). These results indicate that *P. aeruginosa* and *S. aureus* secrete a soluble enhancer(s) of syndecan-1 shedding, and suggest that this property may be specific for certain bacterial species.

Enhancement of syndecan-1 shedding by P. aeruginosa is rapid and dose-dependent, and various host cells are affected

Because all tested strains of *P. aeruginosa* enhanced syndecan-1 shedding, we focused our subsequent studies on this Gram negative bacteria. The clinical blood isolate, BL2, showed the highest level of enhancement (~14-fold, Fig. 1) and was therefore chosen for further studies. As shown in figure 2, enhancement of epithelial syndecan-1 shedding by BL2 culture supernatant was potent (4-fold increase over control levels with 5% supernatant, Fig. 2A), rapid (6-fold increase by 2 h, Fig. 2B), excessive (11-fold increase by 20 h, Fig. 2B) and dependent on the concentration of BL2 supernatant (Fig. 2A). In contrast to constitutive shedding, which maintains constant levels of cell surface syndecan-1, *P. aeruginosa*-enhanced shedding reduced the amount of surface syndecan-1 (90% reduction at 20% supernatant, Fig. 2A). Under the test conditions, responding NMuMG epithelial cells remained viable.

To examine whether *P. aeruginosa* can affect syndecan-1 shedding by other epithelial cell types and host cells, we tested the effects of BL2 culture supernatant on LA-4 lung and C127 mammary gland epithelia, and NIH3T3 fibroblasts. As shown in figure 3, BL2 culture supernatants augmented syndecan-1 shedding by more than 5-fold for all cell types tested. The extent of shedding enhancement was highest with NMuMG epithelia (~13-fold), followed by C127 epithelia (~10-fold), LA-4 epithelia (~8-fold) and NIH3T3 fibroblasts (~5-fold). These results demonstrate that although the physiological target cell type of *P. aeruginosa*, the epithelia, responds most

extensively to shedding enhancement by *P. aeruginosa* supernatant, other host cells such as fibroblasts can also respond.

Identification of the P. aeruginosa syndecan-1 shedding enhancer as LasA

Our experiments testing purified elastase (pseudolysin) and LPS, two major virulence factors of *P. aeruginosa*, failed to demonstrate that these virulence factors are involved in enhancement of syndecan-1 shedding. Thus, to better understand the mechanism behind shedding enhancement by *P. aeruginosa*, we next performed experiments that would provide a general characterization of the *P. aeruginosa* syndecan-1 shedding enhancer. First, we examined whether the activity is susceptible to proteinase K treatment to determine whether the enhancer is a protein. BL2 supernatant was pre-treated with proteinase K and then tested for enhancement of syndecan-1 shedding. As can be seen in figure 4, proteinase K treatment abolished the activity of *P. aeruginosa* supernatant. We next fractionated the crude supernatant with molecular weight cutoff (MWCO) spin tubes to obtain a rough estimate of the enhancer's size. Using 3, 10, 30 and 100 kDa MWCO tubes, we found that the size of the shedding enhancer is larger than 10 kDa but smaller than 30 kDa (Fig. 4, denoted by *). These results suggest that the syndecan-1 shedding enhancer is a 10-30 kDa protein.

Based on these properties of the shedding enhancer, proteins in the supernatant were collected by 80% ammonium sulfate precipitation and fractionated by Bio-Gel P-30 (fractionation range=2.5-40 kDa) gel chromatography in an effort to determine the identity of the enhancer. Fractions obtained from gel chromatography were assayed for shedding enhancement and then analyzed by SDS-PAGE. As shown in figure 5, the shedding enhancing activity was isolated in one peak and two fractions, 12 and 13. Analysis of the active and inactive fractions by 12% SDS-PAGE and Coomassie staining revealed the presence of a single, major 20 kDa band in the active, but not in the inactive, fractions (Fig. 5-insert). To determine the identity of the 20 kDa protein, N-terminal sequencing was performed. The first 10 amino acid sequence of the 20 kDa protein matched perfectly with mature LasA protein (Table 1), a known virulence factor of *P. aeruginosa*.

The hypothesis that LasA is the syndecan-1 shedding enhancer of *P. aeruginosa* was tested by fractionating the partially purified active peak obtained from Bio-Gel P-30 gel filtration by immunoaffinity chromatography using mouse polyclonal anti-LasA IgGs covalently coupled to a cross-linked agarose resin. The rationale behind this experiment was that if LasA is indeed the shedding enhancer, then the active component in the partially purified material will be bound to the affinity column, and shedding activity will only be seen with the specifically bound fractions and not with the flow through or wash fractions. As shown in figure 6, the specifically bound eluate (EL), but not the flow through (FT) and wash (WSH) fractions, enhanced syndecan-1 shedding by NMuMG cells. The inactive flow through fraction contained the contaminating smear seen in the active fractions partially purified by gel chromatography, and the eluate fraction contained the highly purified 20 kDa LasA protein (Fig. 6-insert). Furthermore, similar to results obtained with crude *P. aeruginosa* supernatants, purified LasA enhanced syndecan-1 shedding by various host cells (Fig. 7) and did not affect steady-state mRNA levels of syndecan-1 (data not shown). Taken together, these results indicate that LasA is the syndecan-1 shedding enhancer of *P. aeruginosa*, and that LasA can be highly purified by consecutive steps of ammonium sulfate precipitation, Bio-Gel P-30 gel filtration, and anti-LasA IgG immunoaffinity chromatography.

LasA enhances syndecan shedding by stimulating the host cell's shedding mechanism

To begin to elucidate the mechanism involved in syndecan-1 shedding enhancement by *P. aeruginosa* LasA, we first examined the macrostructure of shed syndecan-1 ectodomains. Conditioned media from NMuMG cells cultured to confluency (constitutively shed) and from NMuMG cells stimulated with purified LasA or crude *P. aeruginosa* supernatant were subjected to DEAE ion exchange chromatography to obtain partially purified samples of syndecan-1. These undigested samples were directly analyzed by Western immunoblotting (Fig. 8, lanes 1-3) or digested by heparitinase and chondroitin sulfate ABC lyase, and then analyzed by immunoblotting (Fig. 8, lanes 4-6) to determine the size of shed syndecan-1 core proteins. Similar to the constitutively shed syndecan-1 ectodomain (lane 1),

syndecan-1 ectodomains obtained from both purified LasA (lane 2) and crude supernatant (lane 3) conditioned media were intact proteoglycans decorated with glycosaminoglycans as evident from the smear of the immunologically detected syndecan-1 ectodomains. Interestingly, the size of the syndecan-1 core protein stimulated to shed by both purified LasA (lane 5) and crude *P. aeruginosa* supernatant (lane 6) was identical to that of the constitutively shed core protein (lane 4) by SDS-PAGE analysis.

Because the macrostructure of the shed syndecan-1 ectodomain suggested that LasA enhances syndecan-1 shedding by a mechanism similar to that of the host cell's shedding mechanism, effects of a hydroxamate derivative (BB1101), PKC antagonist (bisindolylmaleimide I) and protein tyrosine kinase (PTK) inhibitors (genistein, Tyrphostin A25) were tested. Genistein (47) and Tyrphostin A25 (48) inhibit PTKs by competing for binding with ATP and tyrosine residues to PTKs, respectively. These general PTK inhibitors inhibit syndecan-1 and -4 shedding stimulated by all known agonists such as EGF, thrombin, sphingomyelinase, ceramide and stress conditions (e.g. heat, hyperosmolarity, mechanical shear) whereas the antagonistic effect of the PKC inhibitor, bisindolylmaleimide I, is restricted to syndecan-1 and -4 shedding induced by EGF, thrombin and phorbol esters (16,19). Hydroxamate derivatives inhibit the activity of the putative cleaving enzyme by chelating its active site zinc atom (49). Thus, general PTK inhibitors are inhibitors of regulated syndecan shedding whereas hydroxamate derivatives are inhibitors of both regulated and constitutive shedding. As shown in Table 2, when co-incubated, BB1101 and Tyrphostin A25 inhibited both purified LasA- and *P. aeruginosa* supernatant-enhanced syndecan-1 shedding by more than 70% and 60%, respectively, at the highest concentration tested. Genistein also partially inhibited enhanced shedding (~45%), but bisindolylmaleimide I did not affect syndecan-1 shedding enhanced by LasA and crude *P. aeruginosa* supernatant. However, when the kinase inhibitors and BB1101 were pre-incubated with purified LasA and removed from the samples prior to incubation with NMuMG cells, none of them inhibited enhanced syndecan-1 shedding. Taken together, these results indicate that the PTK inhibitors and BB1101 are acting on the host cell, and that LasA is enhancing syndecan-1 shedding via activation of the host cell's shedding mechanism.

DISCUSSION

Enhancement of Host Effector Shedding by Pathogenic Bacteria

The current emergence of antibiotic resistant strains has been driven mainly by overuse of antibacterial agents aimed at inhibiting essential aspects of bacterial metabolism, such as cell wall and protein synthesis, placing selective pressure on the bacteria to develop resistance to these agents. Thus, to prevent development of resistance, it may be ideal to develop prophylactic and therapeutic agents that target specific host-pathogen interactions involved in bacterial pathogenesis. Enhancement of host effector shedding may be one such target of the pathogenesis cascade. Many bacterial pathogens as diverse as *P. aeruginosa*, *S. aureus*, *S. epidermidis*, *E. coli*, *Serratia marcescens* and *Listeria monocytogenes* have the ability to enhance shedding of host surface effectors, such as CD14, TNF-α and IL-6 receptor (30-32). These bacteria are not only distinguished by their cell wall characteristics and sites of colonization, but also by their arsenal of genetically distinct virulence factors. Thus, the shared ability to enhance shedding of host molecules indicates functional convergence and suggests that shedding enhancement is a critical aspect of bacterial pathogenesis. We report here that two major opportunistic bacterial pathogens, *P. aeruginosa* and *S. aureus*, secrete potent enhancers of syndecan-1 shedding. The ability to augment shedding of syndecan-1 appears to be specific for these bacterial species since several other Gram negative and positive bacteria failed to do so. The finding that evolutionarily diverse bacteria, such as *P. aeruginosa* and *S. aureus*, can enhance syndecan-1 shedding suggests that this activity may be critical for their pathogenesis at target host sites common to both. In this regard, it is interesting to note that *P. aeruginosa* and *S. aureus* are the dominant pathogens in cystic fibrosis and burn patients, and that syndecan-1 is the major syndecan of target cell types at these tissue sites, the lung epithelia and keratinocytes, respectively.

Mechanism of syndecan-1 shedding enhancement by LasA

Although we have not yet identified the *S. aureus* shedding enhancer, we have found that the syndecan-1 shedding enhancer of *P. aeruginosa* is the 20 kDa mature LasA protein. LasA is secreted as a precursor protein of approximately 40 kDa,

which is then processed to the mature 20 kDa form by unknown mechanisms (50,51). Mature LasA is a zinc metalloendopeptidase with strong staphylolytic and weak elastolytic activities (52,53). The alternative name of LasA, staphylolysin, is derived from its ability to lyse staphylococcal cells. Because of its elastolytic activity, LasA was first thought of as *P. aeruginosa* elastase. It is now known that the role of LasA in elastolysis is to render the insoluble elastin substrate more susceptible to cleavage by the real *P. aeruginosa* elastase and other enzymes with elastolytic activities (50,54).

The capacity of LasA to hydrolyze protein substrates such as elastin, albeit weak, suggests that LasA may enhance syndecan-1 shedding by direct cleavage of the proteoglycan. However, several lines of evidence indicate that this is not the mechanism of syndecan-1 shedding enhanced by LasA. First, the proteolytic specificity of LasA is rather restricted in that potential substrates are those with Gly in the P1 and P2 positions, Gly, Ala or Phe at P1' and apolar residues in the flanking sequences (52). This tight requirement is exemplified by the fact that elastin, with several GGA, GGG and GGF motifs, and *S. aureus*, with the pentaglycine peptide moiety in its cell wall peptidoglycan, are hydrolyzed by LasA, but casein, without these motifs, is not. Syndecan-1 also does not contain these LasA susceptible motifs. Second, our results show that the size of the core protein shed by LasA and endogenous mechanisms of the host cell is identical, and that PTK and hydroxamate inhibitors of LasA-mediated syndecan-1 shedding are inhibiting shedding by acting on the responding host cells and not on LasA. The PTK inhibitors and the hydroxamate derivative, BB1101, can inhibit shedding only when LasA, the reagents and host cells are co-incubated, and not when the inhibitors are pre-incubated with LasA and removed prior to incubation of the pre-treated LasA with host cells. Furthermore, hydroxamate inhibitors are thought to be specific for the HEXXH zinc-binding catalytic domain of metalloendopeptidases, such as the matrix metalloproteinases (49), but the zinc-binding motif of LasA is HXH. Taken together, these findings indicate that LasA enhances shedding of syndecan-1 by activating the host cell's shedding mechanism.

Implications of LasA-enhanced syndecan-1 shedding in P. aeruginosa *pathogenesis*

Based on the finding that LasA is a virulence factor in animal models of corneal (58) and lung infections (59,60), two tissue sites where syndecan-1 is the predominant syndecan on target epithelia, our working hypothesis for the newly identified function of LasA to enhance shedding of syndecan-1 is that it is a pathogenic activity. There are several ways syndecan-1 shedding may contribute to *P. aeruginosa* pathogenesis. First, host cell invasion by many intracellular pathogens has been found to require signaling by PTKs, ceramide, and more downstream signaling components such as MAP kinases (61-64). These signal transducers are also potent inducers of syndecan shedding (16,19), suggesting that shedding of syndecans from the cell surface accompanies host cell invasion by certain microbial pathogens. Although *P. aeruginosa* is not traditionally thought of as an intracellular pathogen, increasing number of studies have reported that *P. aeruginosa* is internalized by host cells (62,65-68).

Furthermore, *P. aeruginosa* invasion is blocked by PTK inhibitors (62,65), which we have identified as specific inhibitors of LasA-mediated syndecan-1 shedding. Taken together, these correlations suggest that syndecan-1 shedding enhanced by LasA may be an important mediator of *P. aeruginosa* invasion. However, it is important to mention that the role of syndecan shedding in bacterial internalization is unknown, and the importance of host cell invasion in *P. aeruginosa* pathogenesis is still controversial.

Second, our results show that enhancement of syndecan-1 shedding by *P. aeruginosa* not only dramatically increases the amount of soluble ectodomains, but is also accompanied by a significant decrease in the level of cell surface syndecan-1. This property may be pathologically significant since in a previous study, we have found that expression of syndecan-1 is essential in maintaining the normal phenotype of simple epithelia (69). Antisense induced depletion of cell surface syndecan-1 altered cell morphology and organization, expression of other adhesion molecules like E-cadherin and β1 integrins, and anchorage-dependent growth characteristics in NMuMG cells. Because highly polarized epithelia is thought of as an effective barrier against microbial colonization (45,46), the concomitant decrease of cell surface syndecan-1 levels observed during LasA-enhanced shedding can potentially enhance *P. aeruginosa* colonization by altering the morphology of target epithelia, disrupting

the polarity of the epithelial barrier and exposing intercellular, basolateral and subepithelial adhesive components.

Alternatively, syndecan-1 shedding enhanced by LasA may contribute to *P. aeruginosa* pathogenesis by interfering with host defense mechanisms. For example, HS chains of shed syndecan-1 ectodomains can bind and modulate the activity of a plethora of host defense effectors such as chemokines, cytokines and proteases (36). Furthermore, we have found in a separate study that shed syndecan-1 ectodomains can inhibit the antibacterial activity of Pro/Arg-rich antimicrobial peptides by binding to the peptides and preventing them from interacting with target bacterial cells (unpublished results). These potentially pro-pathogenic activities indicate that shed syndecan-1 ectodomains may act as host-derived effectors in the virulence mechanism mediated by LasA. To further decipher the role of enhanced shedding in *P. aeruginosa* pathogenesis, we are currently evaluating the role of syndecan-1 shedding in murine models of bacterial infection using specific agonists and antagonists of syndecan shedding, and also using mice lacking syndecan-1 or overexpressing a constitutively shed form of syndecan-1.

Table 1

Amino Terminal Sequencing of the 20 kDa Putative Syndecan-1 Shedding Enhancer Isolated from *P. aeruginosa*

The 20 kDa shedding enhancer, partially purified by ammonium sulfate precipitation and gel chromatography, was subjected to 12% SDS-PAGE and electrophoretically transferred to Problott PVDF membrane for 1 h at 200 mA using CAPS transfer buffer (10 mM CAPS, pH 11, 10% MeOH in de-ionized H_2O). The 20 kDa band was visualized by Coomassie Brilliant blue R-250 staining, destained, washed extensively with de-ionized water, excised from the membrane and sequenced directly using an Applied Biosystems 477A protein sequencer at the Department of Physiology Core Facility at Tufts University Medical School.

	5 10
20 kDa protein	A P P S N L M Q L P
Mature LasA	A P P S N L M Q L P

Table 2

Effects of Inhibitors on Syndecan-1 Shedding
Enhanced by Purified LasA and *P. aeruginosa* supernatant

Confluent cultures of NMuMG cells in 96 well plates were incubated with purified LasA (5 μg/ml) or BL2 supernatant (20%, v/v) with or without the hydroxamate and signaling inhibitors for 6h at 37°C. Alternatively, purified LasA was pre-incubated with the inhibitors for 3 h at 37°C, centrifuged against a 10 kDa MWCO membrane to remove the inhibitors and then incubated with NmuMG cells for 6h at 37°C (ND=not determined).

2. Inhibitors	II. SYNDECAN-1 ECTODOMAIN SHED (mean % of controls ± SD)	
Co-incubated	Purified LasA	*P. aeruginosa* supernatant
None	100.0 ± 9.5	100.0 ± 8.7
BB1101		
2 μM	28.5 ± 2.7	22.1 ± 10.3
0.2 μM	40.2 ± 8.7	33.6 ± 23.7
Bisindolylmaleimide		
1 μM	81.8 ± 4.4	88.7 ± 1.8
0.2 μM	85.0 ± 18.8	111.6 ± 13.4
Genistein		
10 μg/ml	54.4 ± 22.7	55.6 ± 14.1
0.2 μg/ml	74.0 ± 4.4	75.9 ± 8.9
Tyrphostin		
10 μg/ml	36.5 ± 5.3	31.6 ± 11.61
0.2 μg/ml	78.3 ± 12.6	80.5 ± 6.5
Pre-treated		
None	100.0 ± 9.5	ND
BB1101 (2 μM)	89.6 ± 11.6	ND
Bisindolylmaleimide (1μM)	81.5 ± 15.5	ND
Genistein (10 μg/ml)	98.2 ± 1.3	ND
Tyrphostin (10 μg/ml)	105.5 ± 9.3	ND

Example 2

Procedure for *In Vivo* Inhibition of Sydecan-1 Shedding Examining the Effects of Heparin (mimics shed syndecan-1 ectodomains) and BB1101 (inhibits shedding).

1) 7d old mouse pups were pre-inoculated intranasally with 20 μM BB1101. One hour later, mice were inoculated intranasally with approximately $2x10^9$ cfus of *P. aeruginosa* strain PAO1 in 7μl TSB.

2) For animals with heparin, mice were inoculated intranasally with $2x10^9$ cfus of bacteria in 7μl of TSB containing 5 μg/ml heparin.

3) Both set of animals were processed the following day for lung (pneumonia) and spleen (bacteremia) colony counts.

Condition	Lung Colonization (mean cfus/mg tissue+ geometric mean)	Bacteremia (spleen)	Mortality
Bacteria only	$1.1 \times 10^6 \pm 3.3 \times 10^5$	$1.4 \times 10^5 \pm 3.0 \times 10^4$	4/10
+ 20 μM BB1101	$4.3 \times 10^4 \pm 6.3 \times 10^3$	695.0 ± 437.0	0/5
+ 5 μg/ml heparin	$3.6 \times 10^7 \pm 3.7 \times 10^6$	$7.3 \times 10^5 \pm 3.2 \times 10^5$	6/7
+ 5 μg/ml synd-1	$1.4 \times 10^6 \pm 1.0 \times 10^6$	$3.9 \times 10^5 \pm 1.6 \times 10^5$	5/7

These experiments show that *Pseudomonas aeruginosa*-enhanced shedding of syndecan-1 promotes lung infection by this pathogen. Creating an environment which mimics excess shedding of syndecan-1 (heparin, purified syndecan-1 ectodomain) further promotes lung colonization whereas inhibition of shedding (BB101) prevents lung infection.

Results from Infection Experiments

Bacterium: *Pseudomonas aeruginosa* (strain PAO1)

Mouse: 7-d old Syndecan-1 null ("KO") or wild type ("WT") in C57/BL or Balb/c backgrounds

Intranasal Inoculum: $2x10^9$ cfus in 7 µl TSB

Incubation: 24 hours at room temperature

Mouse Strain	Lung Colonization (mean cfus/mg tissue + geometric mean)	Bacteremia (spleen)	Mortality
C57/BL-WT (n=6)	$9.9x10^5 \pm 2.61x10^4$	$6.8x10^4 \pm 1.1x10^4$	1/6
C57/BL-KO (n=4)	19.0 ± 1.4	2.0 ± 0.3	0/4
Balb/c-WT (n= 8)	$1.6x10^6 \pm 1.0x10^5$	$9.5x10^4 \pm 1.3x10^3$	8/18
Balb/c-KO (n=4)	260.0x4.5	167.0 ± 0.9	0/4

These results indicate that mice lacking syndecan-1 are resistant to *Pseudomonas aeruginosa* lung infection relative to their wild type controls.

Bacterium: *Pseudomonas aeruginosa* (strain PAO1)

Inoculum: intraperitoneal

Incubation: 24 hours at room temperature

Mouse Strain	Lung Colonization (mean cfus/mg tissue + geometric mean)	Bacteremia (spleen)	Mortality
Balb/c-WT $3.3x10^7$ cfus (n=6)	$1.4x10^6 \pm 5.5x10^5$	$6.8x10^4 \pm 1.2x10^4$	3/6
C57/BL-KO $1.7x10^7$ cfus (n=3)	$1.1x10^6 \pm 5.5x10^5$	$6.7x10^4 \pm 1.7x10^4$	2/3

These results indicate that resistance is specific to lung infection in that there is no difference in pathogenesis with intraperitoneal inoculation. These

results further indicate that epithelial syndecan-1 can promote infection whereas lack of syndecan-1 can provide resistance.

Example 3

Effects of PTK Inhibitors on
Syndecan-1 Ectodomain Shedding enhanced by Purified LasA

Condition	PTK Specificity	Syndecan-1 Ectodomain Shed (mean % of control ± SD)
LasA (5μg/ml)		100.0 ± 18.4
+herbimycin A	Src family PTKs	
5 μM		40.3 ± 13.7
1 μM		54.0 ± 10.4
+PP2 (calbiochem)	Fyn & LCK	
250 μM		91.8 ± 13.2
50 μM		120.3 ± 18.7
+AG490 (calbiochem)	JAK2	
1 μM		120.6 ± 13.9
200 nM		97.1 ± 11.5

Confluent cultures of NMuMG cells in 96 well plates were incubated with purified LasA (5 μg/ml) with or without the PTK inhibitors for 6 h at 37°C. Extent of shedding was quantified by the dot immunoblotting method.

These results show that *Pseudomonas aeruginosa* enhanced shedding requires Src PTK activity.

REFERENCES

1. Hooper, N. M., Karran, E. H., and Turner, A. J. (1997) *Biochem. J.* **321,** 265-279
2. Rose-John, S., and Heinrich, P. C. (1994) *Biochem. J.* **300,** 281-290
3. Arribas, J., Coodly, L., Vollmer, P., Kishimoto, T. K., Rose-John, S., and Massague, J. (1996) *J. Biol. Chem.* **271,** 11376-11382
4. Kahn, J., Ingraham, R. H., Shirley, F., Migaki, G. I., and Kishimoto, T. K. (1994) *J. Cell Biol.* **125,** 461-470
5. Bazil, V., and Strominger, J. L. (1991) *J. Immunol.* **147,** 1567-1574
6. Dempsey, P. J., Meise, K. S., Yoshitake, Y., Nishikawa, K., and Coffey, R. J. (1997) *J. Cell Biol.* **138,** 747-758
7. Mohler, K. M., Sleath, P. R., Fitzner, J. N., Cerretti, D. P., Alderson, M., Kerwar, S. S., Torrance, D. S., Otte-Evans, C., Greenstreet, T., Weerawarna, K., Kronheim, S. R., Petersen, M., Gerhart, M., Kozlosky, C. J., March, C. J., and Black, R. A. (1994) *Nature* **370,** 218-220
8. McGeehan, G. M., Becherer, J. D., Bast, R. C. J., Boyer, C. M., Champion, B., Connolly, K. M., Conway, J. G., Furdon, P., Karp, S., Kidao, S., McElroy, A. B., Nichols, J., Pryzwansky, K. M., Schoenen, F., Sekut, L., Truesdale, A., Verghjese, M., Warner, J., and Ways, J. P. (1994) *Nature* **370,** 558-561
9. Engelmann, H., Novick, D., and Wallach, D. (1990) *J. Biol. Chem.* **265,** 1531-1536
10. Brakebusch, C., Varfolomeev, E. E., Batkin, M., and Wallach, D. (1994) *J. Biol. Chem.* **269,** 32488-32496
11. Mullberg, J., Durie, F. H., Otten-Evans, C., Alderson, M. R., Rose-John, S., Cosman, D., Black, R. A., and Mohler, K. M. (1995) *J. Immunol.* **155,** 5198-5205
12. Tanaka, M., Suda, T., Takahashi, T., and Nagata, S. (1995) *EMBO J.* **14,** 1129-1135
13. Pandiella, A., and Massague, J. (1991) *J. Biol. Chem.* **266,** 5769-5773
14. Jones, S. A., Novick, D., Horiuchi, S., Yamamoto, N., Szalai, A. J., and Fuller, G. M. (1999) *J. Exp. Med.* **189,** 599-604
15. Manna, S. K., and Aggarwal, B. B. (1998) *J. Biol. Chem.* **273,** 33333-33341

16. Subramanian, S. V., Fitzgerald, M. L., and Bernfield, M. (1997) *J. Biol. Chem.* **272,** 14713-14720

17. Frey, M., Appenheimer, M. M., and Evans, S. S. (1997) *J. Immunol.* **158,** 5424-5434

18. Bennet, T. A., Lynam, E. B., Sklar, L. A., and Rogelj, S. (1996) *J. Immunol.* **156,** 3093-3097

19. Fitzgerald, M. L., Park, P. W., Wang, Z., Chun, J.-S., Murphy, G., and Bernfield, M. (1999) *J. Cell Biol.* **submitted**

20. Isberg, R. R., and Leong, J. M. (1990) *Cell* **60,** 861-871

21. Relman, D., Tuomanen, E., Falkow, S., Golenbock, D. T., Saukkonen, K., and Wright, S. D. (1990) *Cell* **61,** 1375-1382

22. Leininger, E., Roberts, M., Kenimer, J. G., Charles, I. G., Fairweather, N., Novotny, P., and Brennan, M. J. (1991) *Proc. Natl. Acad. Sci.* **88,** 345-349

23. Wickman, T. J., Mathias, P., Cheresh, D. A., and Nemerow, G. R. (1993) *Cell* **73,** 309-319

24. Isberg, R. R., and Tran Van Nhieu, G. (1994) *Trends Microbiol.* **2,** 10-14

25. Finlay, B. B., and Cossart, P. (1997) *Science* **276,** 718-725

26. Suter, S. (1994) *Am. J. Respir. Crit. Care Med.* **150,** S118-S122

27. Travis, J., Potempa, J., and Maeda, H. (1995) *Trends Microbiol.* **3,** 405-407

28. Okamoto, T., Akaike, T., Suga, M., Tanase, S., Horie, H., Miyajima, S., Ando, M., Ichinose, Y., and Maeda, H. (1997) *J. Biol. Chem.* **272,** 6059-6066

29. Rietschel, E. T., Kirikae, T., Schade, F. U., Mamat, U., Schmidt, G., Loppnow, H., Ulmer, A. J., Zähringer, U., Seydel, U., Di Padova, F., Schreier, M., and Brade, H. (1994) *FASEB J.* **8,** 217-225

30. Walev, I., Vollmer, P., Palmer, M., Bhakdi, S., and Rose-John, S. (1996) *Proc. Natl. Acad. Sci.* **93,** 7882-7887

31. Vollmer, P., Walev, I., Rose-John, S., and Bhakdi, S. (1996) *Infect. Immun.* **64,** 3646-3651

32. Mattson, E., Van Dijk, H., Verhoef, J., Norrby, R., and Rollof, J. (1996) *Infect. Immun.* **64,** 4351-4355

33. Blum, H., Wolf, M., Enssle, K., Rollinghoff, M., and Gessner, A. (1996) *J. Immunol.* **157,** 1846-1853

34. Hayashi, J., Masaka, T., and Ishikawa, I. (1999) *Infect. Immun.* **67,** 417-420

35. Munk, M. E., Anding, P., Schettini, A. P. M., Cunha, M. G. S., and Kaufman, S. H. E. (1999) *Infect. Immun.* **67,** 423-425

36. Bernfield, M., Götte, M., Park, P. W., Reizes, O., Fitzgerald, M. L., Lincecum, J., and Zako, M. (1999) *Annu. Rev. Biochem.* **in press**

37. Grassmé, H., Gulbins, E., Brenner, B., Ferlinz, K., Sandhoff, K., Harzer, K., Lang, F., and Meyer, T. F. (1997) *Cell* **91,** 605-615

38. Rostand, K. S., and Esko, J. D. (1997) *Infect. Immun.* **65,** 1-8

39. Kato, M., Wang, H., Kainulainen, V., Fitzgerald, M. L., Ledbetter, S., Ornitz, D. M., and Bernfield, M. (1998) *Nat. Med.* **4,** 691-697

40. Kainulainen, V., Wang, H., Schick, C., and Bernfield, M. (1998) *J. Biol. Chem.* **273,** 11563-11569

41. Kim, C. W., Goldberger, O. A., Gallo, R. L., and Bernfield, M. (1994) *Mol. Biol. Cell* **5,** 797-805

42. Krivan, H. C., Roberts, D. D., and Ginsburg, V. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 6157-6161

43. Jalkanen, M., Nguyen, H., Rapraeger, A., Kurn, N., and Bernfield, M. (1985) *J. Cell Biol.* **101,** 976-984

44. Hansen, M. B., Nielsen, S. E., and Berg, K. (1989) *J. Immunol. Methods* **119,** 203-210

45. Kagnoff, M. F., and Eckmann, L. (1997) *J. Clin. Invest.* **100,** 6-10

46. Wick, M. J., Madara, J. L., Fields, B. N., and Normark, S. J. (1991) *Cell* **67,** 651-659

47. Akiyama, T., and Ogawara, H. (1991) *Methods Enzymol.* **201,** 362-370

48. Gazit, A., Yaish, P., Gilon, C., and Levitzki, A. (1989) *J. Med. Chem.* **32,** 2344-2352

49. Wojtowicz-Praga, S. M., Dickson, R. B., and Hawkins, M. J. (1997) *Invest. New Drugs* **15,** 61-75

50. Peters, J. E., and Galloway, D. R. (1990) *J. Bacteriol.* **172,** 2236-2240

51. Kessler, E., Safrin, M., Gustin, J. K., and Ohman, D. E. (1998) *J. Biol. Chem.* **273,** 30225-30231

52. Kessler, E., Safrin, M., Abrams, W. R., Rosenbloom, J., and Ohman, D. E.

(1997) *J. Biol. Chem.* **272,** 9884-9889

53. Gustin, J. K., Kessler, E., and Ohman, D. E. (1996) *J. Bacteriol.* **178,** 6608-6617
54. Galloway, D. R. (1991) *Mol. Microbiol.* **5**(10), 2315-2321
55. Reiland, J., Ott, V. L., Lebakken, C. S., Yeaman, C., McCarthy, J., and Rapraeger, A. C. (1996) *Biochem. J.* **319,** 39-47
56. Kinnunen, T., Kaksonen, M., Saarinen, J., Kalkkinen, N., Peng, H. B., and Rauvala, H. (1998) *J. Biol. Chem.* **273,** 10702-10708
57. Ott, V. L., and Rapraeger, A. C. (1998) *J. Biol. Chem.* **273,** 35291-35298
58. Preston, M. J., Seed, P. C., Toder, D. S., Iglewski, B. H., Ohman, D. E., Gustin, J. K., Goldberg, J. B., and Pier, G. B. (1997) *Infect. Immun.* **65,** 3086-3090
59. Woods, D. E., Cryz, S. J., Friedman, R. L., and Iglewski, B. H. (1982) *Infect. Immun.* **36,** 1223-1228
60. Blackwood, L. L., Stone, R. M., Iglewski, B. H., and Pennington, J. E. (1983) *Infect. Immun.* **39,** 198-201
61. Bliska, J. B., Galán, J. E., and Falkow, S. (1993) *Cell* **73,** 903-920
62. Evans, D. J., Frank, D. W., Finck-Barbançon, V., Wu, C., and Fleiszig, S. M. J. (1998) *Infect. Immun.* **66,** 1453-1459
63. Finlay, B. B., and Falkow, S. (1989) *Microbiol. Rev.* **53,** 210-230
64. Tang, P., Sutherland, C. L., Gold, M. R., and Finlay, B. B. (1998) *Infect. Immun.* **66,** 1106-1112
65. Fleiszig, S. M. J., Zaidi, T. S., and Pier, G. B. (1995) *Infect. Immun.* **63,** 4072-4077
66. Fleiszig, S. M., Evans, D. J., Do, N., Vallas, V., Shin, S., and Mostov, K. E. (1997) *Infect. Immun.* **65,** 2861-2867
67. Hauser, A. R., Fleiszig, S., Kang, P. J., Mostov, K., and Engel, J. N. (1998) *Infect. Immun.* **66,** 1413-1420
68. Pier, G. B., Grout, G., Zaidi, T. S., Olsen, J. C., Johnson, L. G., Yankaskas, J. R., and Goldberg, J. B. (1996) *Science* **271,** 64-67
69. Kato, M., Saunders, S., Nguyen, H., and Bernfield, M. (1995) *Mol. Biol. Cell* **6,** 559-576

70. Shanson D.C. (1986) *Br. J. Hosp. Med.* 35(5):312, 314, 318-320.

71. Wiblin R.T. (1997) Nosocomial pneumonia. In: Wenzel R.P., editor. Prevention and control of nosocomial infections. 3RD ed. Baltimore: Williams and Wilkins; p. 807-819.

72. Van Delden C., et al. (1998) *Emerg. Infect. Dis.* 4(4):551-560.

73. Fergie J.E., et al. (1994) *Clin. Infect. Dis.* (18):390-394.

74. Bergen G.A, et al. (1996) *Infect. Dis. Clin. North Am.* (10):297-326.

75. Dunn M., etal. (1995) *Clinics in Chest Med.* (16):95-109.

76. Pollack M. (1995) *Pseudomonas aeruginosa.* In: Mandell G.L., Benett J.E., Dolin R, editors. Principles and practice of infectious diseases. 4TH ed. New York: Churchill Livingstone; p. 1980-2003.

77. Kluytmans J. (1997) Surgical infections including burns. In Wenzel R.P., editor. Prevention and control of nosocomial infections. 3RD ed. Baltimore: Williams and Wilkins; p. 841-865.

78. Gordon S.M., et al. (1998) *Ann. Thorac. Surg.* (65):95-100.

79. Mendelson M.H., et al. (1994) *Clin. Infect. Dis.* (18):886-895.

80. Govan J.R., et al. (1996) *Microbiol. Rev.* 60:539-574.

81. Pier G.B. (1998) *ASM News* 64(6):339-347.

82. Murray B. E. (1989) *Infect. Dis. Clin. North Am.* 3(3):423-4339.

83. Thornsberry C. (1988) *J. Antimicrob. Chemother.* Apr;21 Suppl. C:9-17.

84. Swartz M.N. (1994) *Proc. Natl. Acad. Sci. U.S.A.* 91(7):2420-2427.

85. Murray B.E. (1991) *J. Infect. Dis.* 163(6):1184-1194.

86. Lacey R.W. , et al. (1986) *J. Antimicrob. Chemother.* Oct;18 Suppl. C:207-214.

87. Turnidge J., et al. (1989) *Med. J. Aust.* 16;150(2):65, 69-72.

88. McManus J. (1999) *B.M.J.* 318(7184):626.

89. Hiramatsu, K., et al (1998) *Am. J. Med.* 104(5A):7S-10S.

90. Hiramatsu K., et al. (1997) *Lancet* 350(9092):1670-3.

91. Sieradzki K., et al. (1999) *N. Engl. J. Med.* 340(7):517-523.

92. Russell A.D. (1997) *J. Appl. Microbiol.* 83(2):155-165.

93. Van Bambeke F., et al. (1999) *Antimicrob. Agents Chemother.* 43(1):41-47.

94. Passador L., et al. (1995) Quorum sensing and virulence gene regulation in *Pseudomonas aeruginosa*. In: Roth J.A., editor. Virulence mechanisms of bacterial pathogens. 2ND ed. Washington: American Society for Microbiology. p.65-78.

95. Richard P., et al.(1994) *J. Infect. Dis.* (170):377-383.

96. Brewer S.C., et al. (1996) *Chest* (109):1019-1029.

All documents mentioned herein are incorporated herein by reference.

The invention has been described in detail with reference to preferred embodiments thereof. However, it will be appreciated that those skilled in the art, upon consideration of this disclosure, may make modifications and improvements within the spirit and scope of the invention.

What is claimed is:

1. A method for treating a bacterial infection comprising:

a) determining whether the bacterium causing the infection is capable of enhancing host cell syndecan-1 shedding; and

b) administering an effective amount of a compound that inhibits syndecan-1 shedding to a subject suffering from said infection, wherein the compound is selected from (a) inhibitors of a bacterial factor responsible for syndecan-1 cleavage, (b) compounds that bind the syndecan and disrupt cleavage, (c) syndecan-1 decoys, and (d) inhibitors of the host cell shedding mechanism, except when the bacterium is *Pseudomonas aeruginosa*, the compound is not genistein or tyrphostin A47.

2. The method of claim 1, wherein the compound is a hydroxamate derivative or a protein tyrosine kinase inhibitor.

3. The method of claim 2, wherein the compound is a hydroxamate derivative.

4. The method of claim 2, wherein the compound is a protein tyrosine kinase inhibitor.

5. The method of claim 4, wherein the protein tyrosine kinase inhibitor is genistein or tyrphostin A25.

6. The method of claim 1, wherein the compound binds the syndecan and disrupts cleavage.

7. The method of claim 6, wherein the compound that binds the syndecan and disrupts cleavage is an antibody.

8. The method of claim 1, wherein the bacterium is *Pseudomonas aeruginosa*.

9. The method of claim 1, wherein the bacterium is *Staphylococcus aureus*.

10. The method of claim 1, wherein the infection is of the respiratory system, the urinary tract, the skin, the eye (cornea), or bloodstream.

11. A method for treating a *Pseudomonas* or *Staphylococcus* lung infection comprising administering an effective amount of a compound that inhibits syndecan-1 shedding to a subject suffering from said infection, wherein the compound is a hydroxamate derivative, a protein tyrosine kinase inhibitor, or an antibody that binds the syndecan and disrupts cleavage, except the compound is not a protein tyrosine kinase inhibitor when the *Pseudomonas* is *Pseudomonas aeruginosa*.

12. A method of identifying a compound useful in the treatment of a bacterial infection comprising contacting a syndecan-1 containing cell with a candidate pharmacological agent and measuring syndecan-1 cleavage.

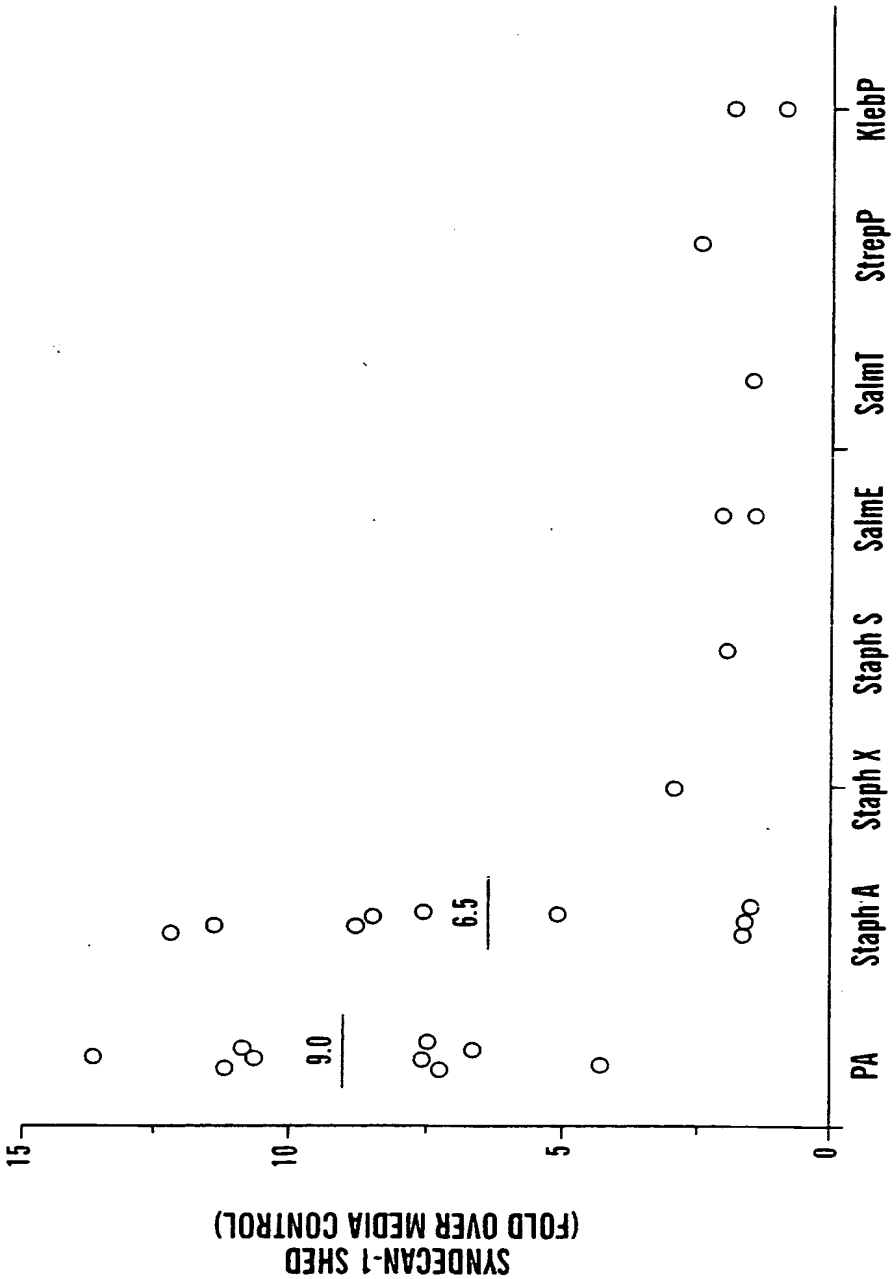
SYNDECAN-1 SHED
(FOLD OVER MEDIA CONTROL)
15
10
5
0
9.0
6.5
PA
Staph A
Staph X
Staph S
SalmE
SalmT
StrepP
KlebP

FIG. 1

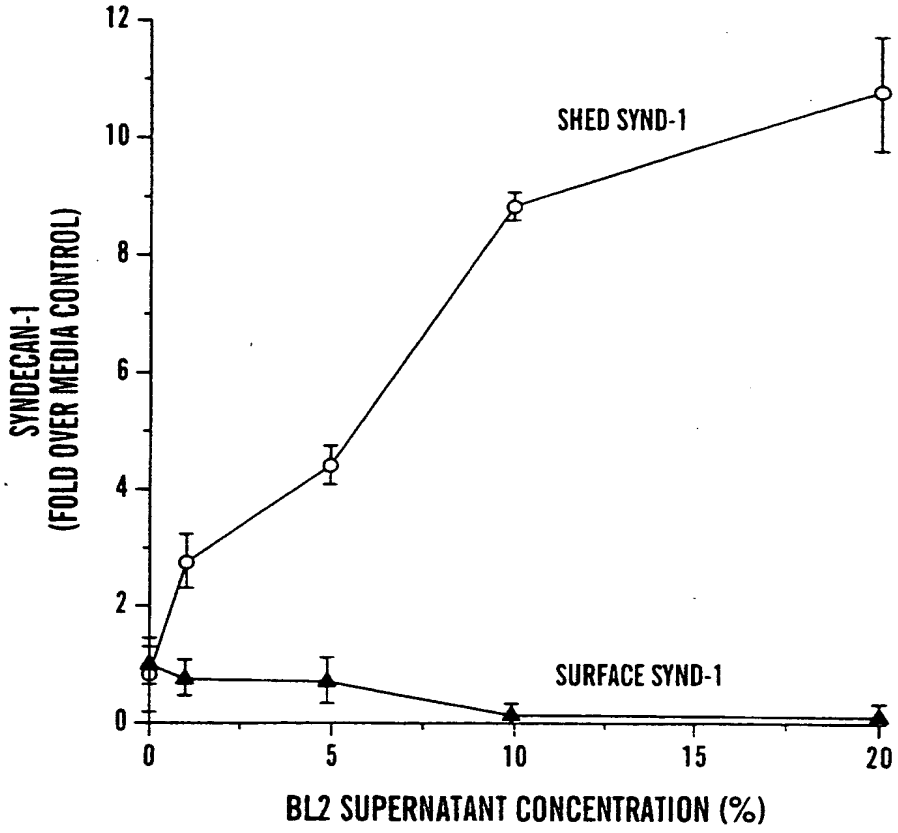
12
10
8
6
4
2
0
SYNDECAN-1
(FOLD OVER MEDIA CONTROL)
SHED SYND-1
SURFACE SYND-1
0
5
10
15
20
BL2 SUPERNATANT CONCENTRATION (%)

FIG. 2A

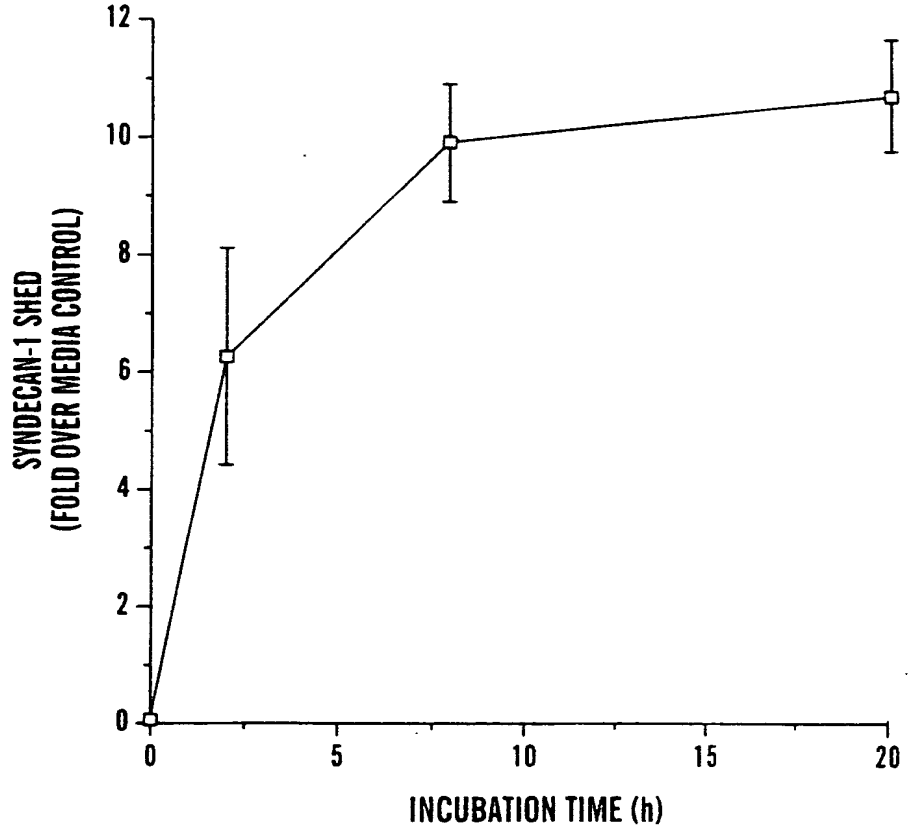
SYNDECAN-1 SHED
(FOLD OVER MEDIA CONTROL)
12
10
8
6
4
2
0
0
5
10
15
20
INCUBATION TIME (h)

FIG. 2B

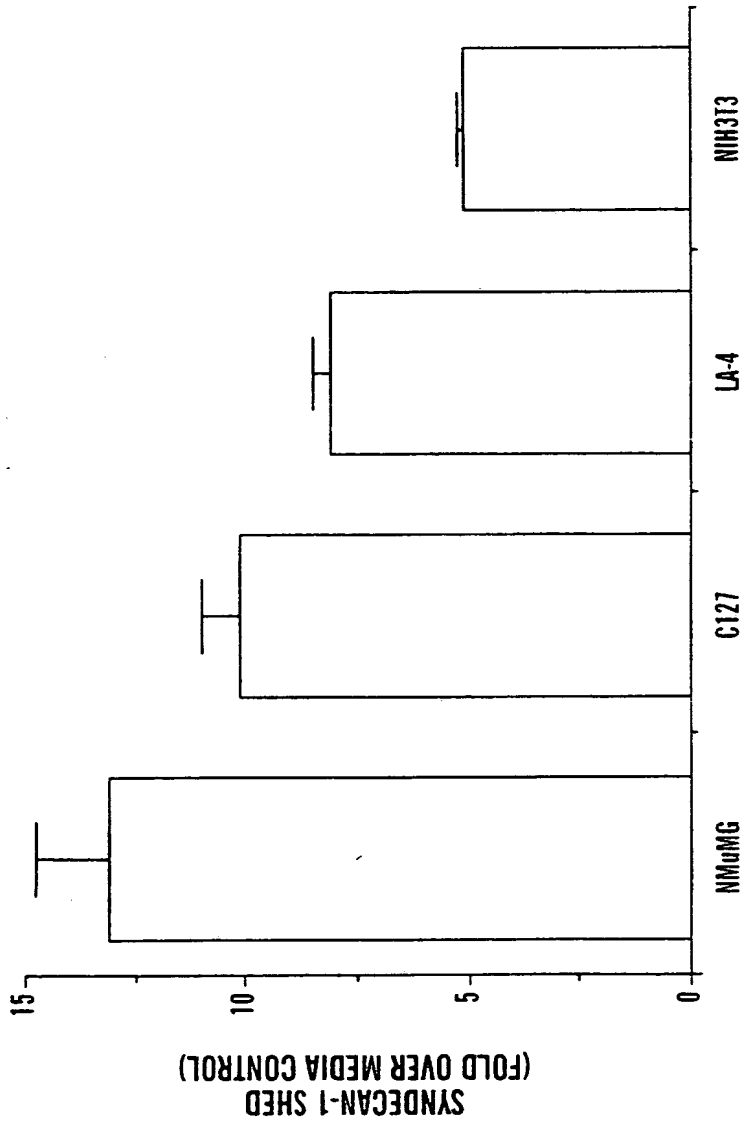
NIH3T3
LA-4
C127
NMuMG
15
10
5
0
SYNDECAN-1 SHED
(FOLD OVER MEDIA CONTROL)

FIG. 3

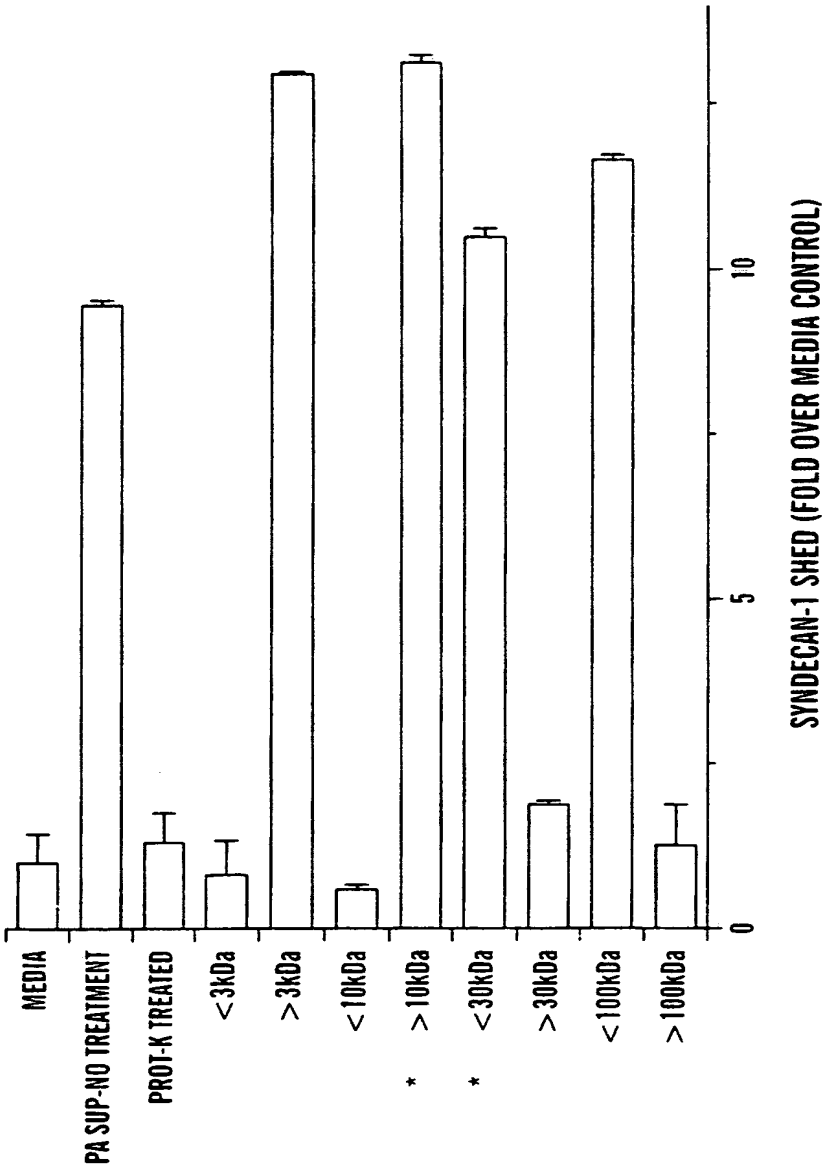
SYNDECAN-1 SHED (FOLD OVER MEDIA CONTROL)
0
5
10
MEDIA
PA SUP-NO TREATMENT
PROT-K TREATED
<3kDa
>3kDa
<10kDa
>10kDa
<30kDa
>30kDa
<100kDa
>100kDa
*
*

FIG. 4

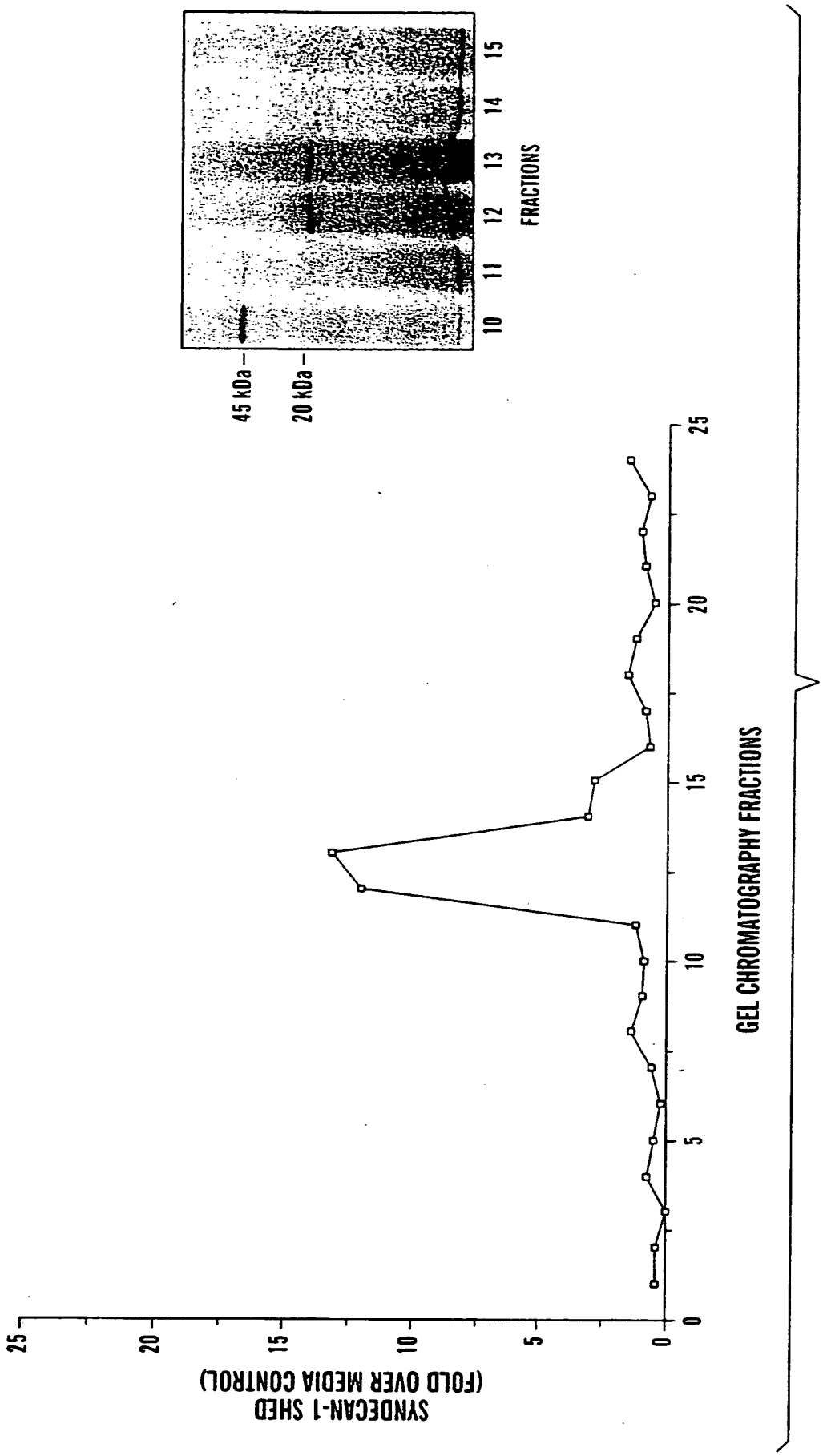
FRACTIONS
10
11
12
13
14
15
45 kDa
20 kDa
SYNDECAN-1 SHED
(FOLD OVER MEDIA CONTROL)
0
5
10
15
20
25
GEL CHROMATOGRAPHY FRACTIONS

FIG. 5

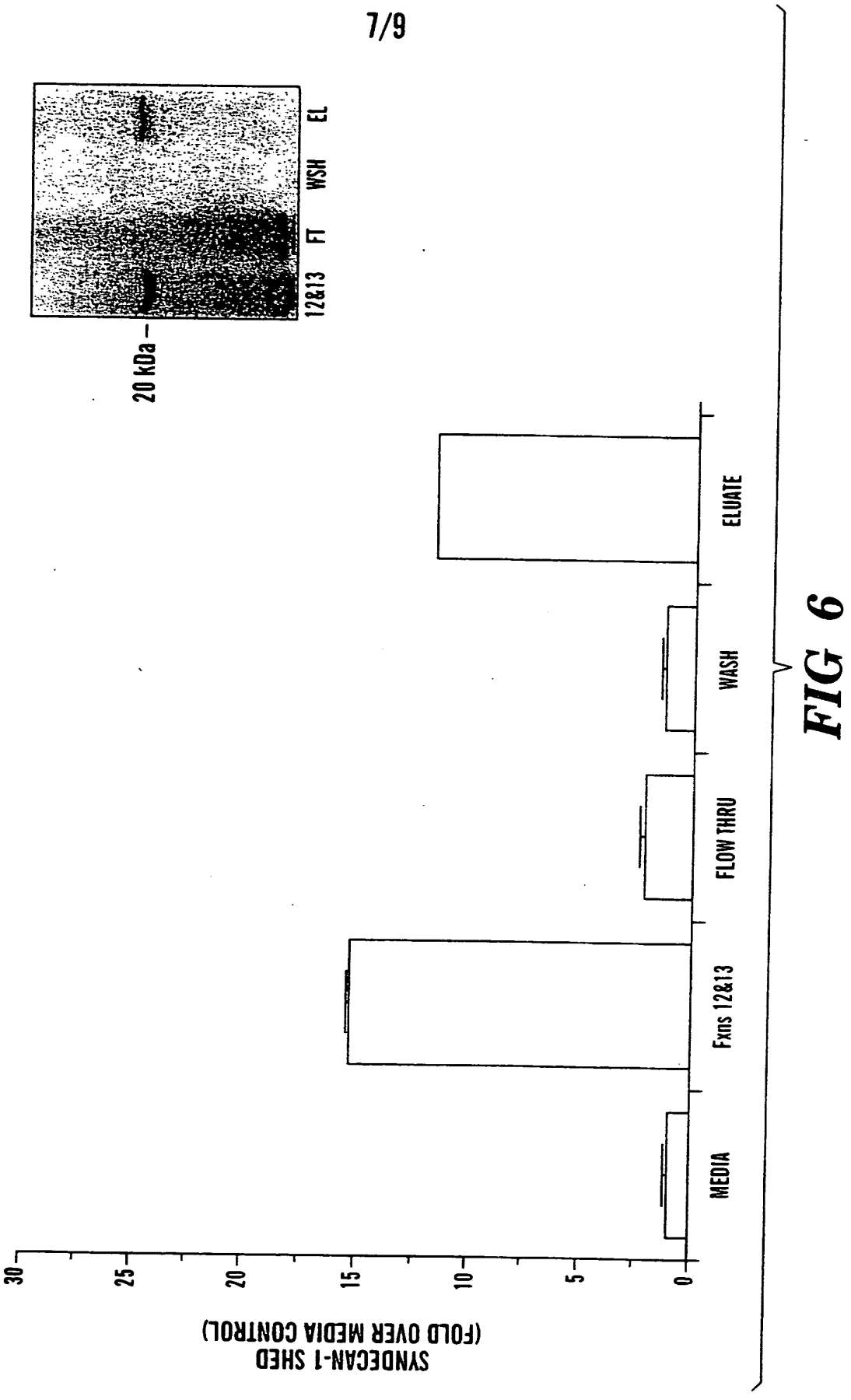
20 kDa
12&13
FT
WSH
EL
SYNDECAN-1 SHED
(FOLD OVER MEDIA CONTROL)
30
25
20
15
10
5
0
MEDIA
Fxns 12&13
FLOW THRU
WASH
ELUATE

FIG 6

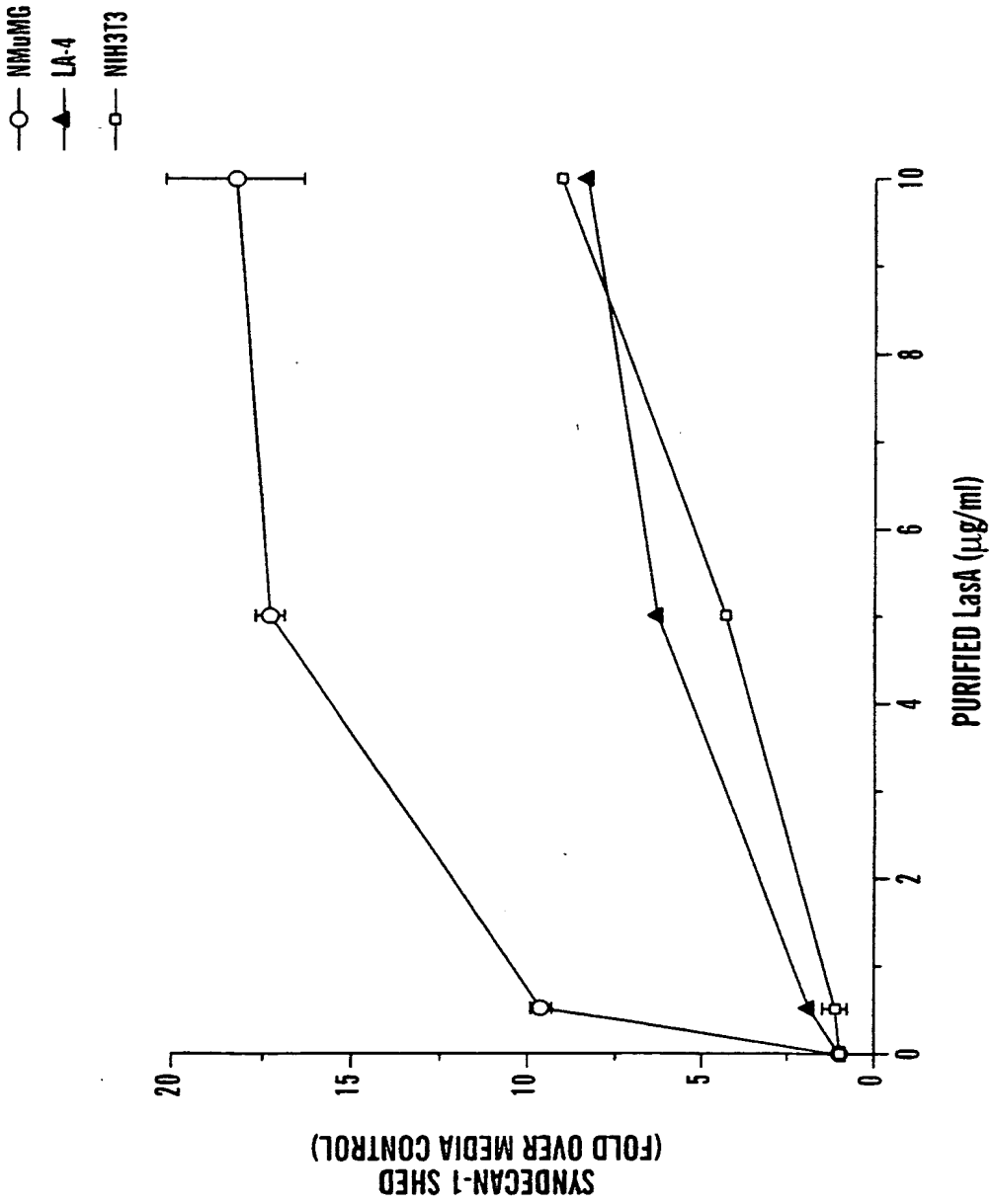
NMuMG
LA-4
NIH3T3
SYNDECAN-1 SHED
(FOLD OVER MEDIA CONTROL)
20
15
10
5
0
0
2
4
6
8
10
PURIFIED LasA (μg/ml)

FIG. 7

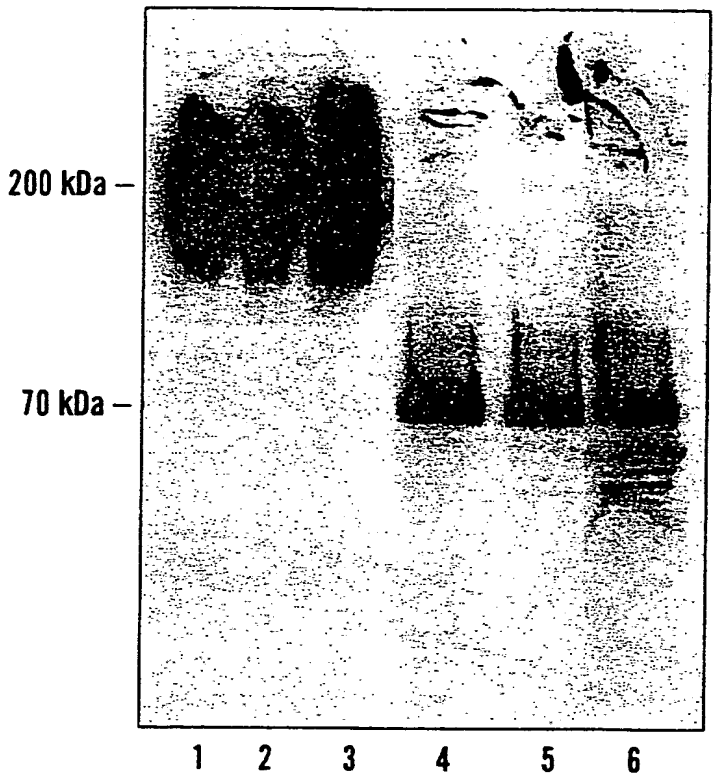

FIG. 8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/24839

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :Please See Extra Sheet.
US CL :424/130.1, 234.1, 243.1, 260.1; 435/4, 32; 530/350, 388.2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/130.1, 234.1, 243.1, 260.1; 435/4, 32; 530/350, 388.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG, MEDLINE, EMBASE, SF ALLSCIENCE, WEST, USPATFULL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Dissertation Abstracts International. Vol. 57, No.12-B, 1997 (Madison, Wisconsin, USA), page 7315. Lebakken, C.S. 'The Role of Syndecan-1 as an Adhesion Receptor in RAJI-S1 Cells.	1-12
Y, P	PARK et al. Syndecan-1 Shedding is Enhanced by LasA, a Secreted Virulence Factor of Pseudomonas aeruginosa. J. Biol. Chem. 04 February 2000, Vol. 275, No. 5, pages 3057-3064, see entire document.	1-12
A	KATO et al. Cell Surface Syndecan-1 on Distinct Cell Types Differs in Fine Structure and Ligand Binding of its Heparin Sufate Chains. J. Biol. Chem. 22 July 1994, Vol. 269, No. 29, pages 18881-18890, see entire document.	1-12

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 17 NOVEMBER 2000	Date of mailing of the international search report 02 JAN 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JENNIFER GRASER Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/24839

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (7):

A61K 39/395, 39/02, 39/085, 39/108; C12Q 1/00, 1/18; C07K 1/00, 16/00

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

REC'D 21 JUN 2001
WIPO PCT

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
701039-48929		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US00/24839	11 SEPTEMBER 2000	10 SEPTEMBER 1999
International Patent Classification (IPC) or national classification and IPC Please See Supplemental Sheet.		
Applicant CHILDREN'S MEDICAL CENTER CORPORATION		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 4 sheets.

   ☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 0 sheets.

3. This report contains indications relating to the following items:

   I ☒ Basis of the report

   II ☐ Priority

   III ☐ Non-establishment of report with regard to novelty, inventive step or industrial applicability

   IV ☐ Lack of unity of invention

   V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

   VI ☐ Certain documents cited

   VII ☐ Certain defects in the international application

   VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
03 APRIL 2001	16 MAY 2001
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Dorthea Lawrence for JENNIFER GRASER Telephone No. (703) 308-0196

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US00/24839

I. Basis of the rep rt

1. With regard to the **elements** of the international application:*

[X] the international application as originally filed

[X] the description:
pages 1-38 , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

[X] the claims:
pages 39-40 , as originally filed
pages NONE , as amended (together with any statement) under Article 19
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

[X] the drawings:
pages 1-9 , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

[X] the sequence listing part of the description:
pages NONE , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language _____ which is:

[ ] the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

[ ] the language of publication of the international application (under Rule 48.3(b)).

[ ] the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

[ ] contained in the international application in printed form.

[ ] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readable form.

[ ] The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] The statement that the information recorded in computer readable form is identical to the writen sequence listing has been furnished.

4. [X] The amendments have resulted in the cancellation of:

[X] the description, pages NONE

[X] the claims, Nos. NONE

[X] the drawings, sheets/~~fig~~ NONE

5. [ ] This report has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

* *Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

** *Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.*

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US00/24839

V. Reas ned statement under Article 35(2) with regard t n velty, inventive step or industrial applicability; citations and explanations supp rting such statement

1. statement

Novelty (N)	Claims	1-12	YES
	Claims	NONE	NO
Inventive Step (IS)	Claims	1-12	YES
	Claims	NONE	NO
Industrial Applicability (IA)	Claims	1-12	YES
	Claims	NONE	NO

2. citations and explanations (Rule 70.7)

Claims 1-12 meet the criteria set out in PCT Article 33(2)-(4), because the prior art does not teach or fairly suggest a method for treating a bacterial infection comprising determining whether the bacterium causing the infection is capable of enhancing host cell syndecan-1 shedding and administering an effective amount of a compound that inhibits syndecan-1 shedding. The prior also does not teach or fairly suggest a method for treating a *Pseudomonas* or *Staphylococcus* lung infetion by administering an effective amount of a compound that inhibits syndecan-1 shedding wherein the compound is not a protein tyrosine kinase inhibitor when the *Pseudomonas* is *P.aeruginosa*. Lastly, the prior art does not teach or fairly suggst a method of identifying a compound useful in the treatment of a bacterial infection comprising contacting a syndecan-1 containing celll with a candidate pharacological agent and measuring syndecan-1 cleavage.

---------- NEW CITATIONS ----------

NONE

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

CLASSIFICATION:

The International Patent Classification (IPC) and/or the National classification are as listed below:
IPC(7): A61K 39/395, 39/02, 39/085, 39/108; C12Q 1/00, 1/18; C07K 1/00, 16/00 and US Cl.: 424/130.1, 234.1, 243.1, 260.1; 435/4, 32; 530/350, 388.2

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

To: DAVID S. RESNICK
NIXON PEABODY LLP
101 FEDERAL STREET
BOSTON, MA 02110

RECEIVED
JAN 0 4 2001
NIXON PEABODY LLP

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

	Date of Mailing *(day/month/year)* 02 JAN 2001
Applicant's or agent's file reference 1039/48929 P	FOR FURTHER ACTION See paragraphs 1 and 4 below
International application No. PCT/US00/24839	International filing date *(day/month/year)* 11 SEPTEMBER 2000
Applicant CHILDREN'S MEDICAL CENTER CORPORATION	

1. [X] The applicant is hereby notified that the international search report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:
The applicant is entitled, if he so wishes, to amend the claims of the international application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the international search report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Facsimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. [ ] The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. [ ] **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

[ ] the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

[ ] no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in rules 90 *bis* 1 and 90 *bis* 3, respectively, before the completion of the technical preparations for international publication.

Within **19 months** from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within **20 months** from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JENNIFER GRASER Dorthea Laurence for Telephone No. (703) 308-0196

Form PCT/ISA/220 (July 1998)*

(See notes on accompanying sheet)

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

To: DAVID S. RESNICK
NIXON PEABODY LLP
101 FEDERAL STREET
BOSTON, MA 02110

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

	Date of Mailing *(day/month/year)* 02 JAN 2001
Applicant's or agent's file reference 1039/48929 P	FOR FURTHER ACTION See paragraphs 1 and 4 below
International application No. PCT/US00/24839	International filing date *(day/month/year)* 11 SEPTEMBER 2000
Applicant CHILDREN'S MEDICAL CENTER CORPORATION	

1. [X] The applicant is hereby notified that the international search report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:
The applicant is entitled, if he so wishes, to amend the claims of the international application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the international search report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Facsimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. [ ] The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. [ ] **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

[ ] the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

[ ] no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in rules 90 *bis* 1 and 90 *bis* 3, respectively, before the completion of the technical preparations for international publication.

Within **19 months** from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within **20 months** from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JENNIFER GRASER Dorothea Lawrence for Telephone No. (703) 308-0196

Form PCT/ISA/220 (July 1998)★ *(See notes on accompanying sheet)*

The following examples illustrate the manner in which amendments must be explained in the accompanying letter:

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."
2. [Where originally there were 15 claims and after amendment of all claims there are 11]:
"Claims 1 to 15 replaced by amended claims 1 to 11."
3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."
4. [Where various kinds of amendments are made]:
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

"Statement under Article 19(1)" (Rule 46.4)

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

The statement should be brief, it should not exceed 500 words if in English or if translated into English.

It should not be confounded with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It should not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

In what language ?

The amendments must be made in the language in which the international application is published. The letter and any statement accompanying the amendments must be in the same language as the international application if that language is English or French; otherwise, it must be in English or French, at the choice of the applicant.

Consequence if a demand for international preliminary examination has already been filed ?

If, at the time of filing any amendments under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the same time of filing the amendments with the International Bureau, also file a copy of such amendments with the International Preliminary Examining Authority (see Rule 62.2(a), first sentence).

Consequence with regard to translation of the international application for entry into the national phase ?

The applicant's attention is drawn to the fact that, where upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

For further details on the requirements of each designated/elected Office, see Volume II of the PCT Applicant's Guide.

NOTES TO FORM PCT/ISA/220

These Notes are intended to give the basic instructions concerning the filing of amendments under Article 19. The Notes are based on the requirements of the Patent Cooperation Treaty and of the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the PCT Applicant's Guide, a publication of WIPO.

In these Notes, "Article", "Rule" and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions, respectively.

INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international publication. Furthermore, it should be emphasized that provisional protection is available in some States only.

What parts of the international application may be amended ?

The claims only.

The description and the drawings may only be amended during international preliminary examination under Chapter II.

When ? **Within 2 months from the date of transmittal of the international search report or 16 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).**

Where not to file the amendments ?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been/is filed, see below.

How ? **Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.**

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Administrative Instructions, Section 205(b)).

What documents must/may accompany the amendments ?

Letter (Section 205(b)):

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confounded with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped), whether

(i) the claim is unchanged;
(ii) the claim is cancelled;
(iii) the claim is new;
(iv) the claim replaces one or more claims as filed;
(v) the claim is the result of the division of a claim as filed.

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To: DAVID S. RESNICK
NIXON PEABODY LLP
101 FEDERAL STREET
BOSTON, MA 02110

PCT

NOTIFICATION OF TRANSMITTAL OF INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Rule 71.1)

Date of Mailing *(day/month/year)*	18 JUN 2001
Applicant's or agent's file reference: 701039-48929	IMPORTANT NOTIFICATION

International application No.	International filing date *(day/month/year)*	Priority Date *(day/month/year)*
PCT/US00/24839	11 SEPTEMBER 2000	10 SEPTEMBER 1999

Applicant
CHILDREN'S MEDICAL CENTER CORPORATION

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices)(Article 39(1))(see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	JENNIFER GRASER Dorothea Lawrence Lee Telephone No. (703) 308-0196

Form PCT/IPEA/416 (July 1992)★

PATENT COOPERATION TRE

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference 1039/48929 P	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
International application No. PCT/US00/24839	International filing date *(day/month/year)* 11 SEPTEMBER 2000	(Earliest) Priority Date *(day/month/year)* 10 SEPTEMBER 1999
Applicant CHILDREN'S MEDICAL CENTER CORPORATION		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the language, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of the sequence listing:

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readable form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (See Box II).

4. With regard to the title,

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the abstract,

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the drawings to be published with the abstract is Figure No. _____

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [X] None of the figures.

INTERNATIO SEARCH REPORT

[redacted]national application No.
PCT/US00/24839

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :Please See Extra Sheet.
US CL :424/130.1, 234.1, 243.1, 260.1; 435/4, 32; 530/350, 388.2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/130.1, 234.1, 243.1, 260.1; 435/4, 32; 530/350, 388.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG, MEDLINE, EMBASE, SF ALLSCIENCE, WEST, USPATFULL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Dissertation Abstracts International. Vol. 57, No.12-B, 1997 (Madison, Wisconsin, USA), page 7315. Lebakken, C.S. 'The Role of Syndecan-1 as an Adhesion Receptor in RAJI-S1 Cells.	1-12
Y, P	PARK et al. Syndecan-1 Shedding is Enhanced by LasA, a Secreted Virulence Factor of Pseudomonas aeruginosa. J. Biol. Chem. 04 February 2000, Vol. 275, No. 5, pages 3057-3064, see entire document.	1-12
A	KATO et al. Cell Surface Syndecan-1 on Distinct Cell Types Differs in Fine Structure and Ligand Binding of its Heparin Sufate Chains. J. Biol. Chem. 22 July 1994, Vol. 269, No. 29, pages 18881-18890, see entire document.	1-12

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 17 NOVEMBER 2000	Date of mailing of the international search report 02 JAN 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JENNIFER GRASER Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/24839

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (7):

A61K 39/395, 39/02, 39/085, 39/108; C12Q 1/00, 1/18; C07K 1/00, 16/00

PATENT COOPERATION TREATY

WO 01/17560
PCT/US00/24839

PCT

From the INTERNATIONAL BUREAU

To:
RESNICK, David, S.
Nixon Peabody LLP
101 Federal Street
Boston, MA 02110
ETATS-UNIS D'AMERIQUE

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

Date of mailing (day/month/year) 15 March 2001 (15.03.01)	
Applicant's or agent's file reference 1039/48929 P	**IMPORTANT NOTICE**

International application No.	**International filing date** (day/month/year)	**Priority date** (day/month/year)
PCT/US00/24839	11 September 2000 (11.09.00)	10 September 1999 (10.09.99)

Applicant
CHILDREN'S MEDICAL CENTER CORPORATION et al

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice:
   AU,US

   In accordance with Rule 47.1(c), third sentence, those Offices will accept the present Notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:
   CA,EP,JP

   The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-bis)).

3. Enclosed with this Notice is a copy of the international application as published by the International Bureau on
   15 March 2001 (15.03.01) under No. WO 01/17560

REMINDER REGARDING CHAPTER II (Article 31(2)(a) and Rule 54.2)

If the applicant wishes to postpone entry into the national phase until 30 months (or later in some Offices) from the priority date, **a demand for international preliminary examination** must be filed with the cornpetent International Preliminary Examining Authority before the expiration of 19 months from the priority date.

It is the applicant's sole responsibility to monitor the 19-month time limit.

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

REMINDER REGARDING ENTRY INTO THE NATIONAL PHASE (Article 22 or 39(1))

If the applicant wishes to proceed with the international application in the **national phase**, he must, within 20 months or 30 months, or later in some Offices, perform the acts referred to therein before each designated or elected Office.

For further important information on the time limits and acts to be performed for entering the national phase, see the Annex to Form PCT/IB/301 (Notification of Receipt of Record Copy) and Volume II of the PCT Applicant's Guide.

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer
J. Zahra

Telephone No. (41-22) 338.83.38

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

INFORMATION CONCERNING ELECTED OFFICES NOTIFIED OF THEIR ELECTION

(PCT Rule 61.3)

To:

RESNICK, David, S.
Nixon Peabody LLP
101 Federal Street
Boston, MA 02110
ETATS-UNIS D'AMERIQUE

Date of mailing (day/month/year) 13 July 2001 (13.07.01)	
Applicant's or agent's file reference 1039/48929 P	**IMPORTANT INFORMATION**

International application No.	**International filing date** (day/month/year)	**Priority date** (day/month/year)
PCT/US00/24839	11 September 2000 (11.09.00)	10 September 1999 (10.09.99)

Applicant
CHILDREN'S MEDICAL CENTER CORPORATION et al

1. The applicant is hereby informed that the International Bureau has, according to Article 31(7), notified each of the following Offices of its election:

   EP :AT,BE,CH,CY,DE,DK,ES,FI,FR,GB,GR,IE,IT,LU,MC,NL,PT,SE
   National :AU,CA,JP,US

2. The following Offices have waived the requirement for the notification of their election; the notification will be sent to them by the International Bureau only upon their request:

   None

3. The applicant is reminded that he must enter the "national phase" **before the expiration of 30 months from the priority date** before each of the Offices listed above. This must be done by paying the national fee(s) and furnishing , if prescribed, a translation of the international application (Article 39(1)(a)), as well as, where applicable, by furnishing a translation of any annexes of the international preliminary examination report (Article 36(3)(b) and Rule 74.1).

   Some offices have fixed time limits expiring later than the above-mentioned time limit. For detailed information about the applicable time limits and the acts to be performed upon entry into the national phase before a particular Office, see Volume II of the PCT Applicant's Guide.

   The entry into the European regional phase is postponed **until 31 months from the priority date** for all States designated for the purposes of obtaining a European patent.

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer:

Juan Cruz

Telephone No. (41-22) 338.83.38

P. ENT COOPERATION TREAT

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF RECEIPT OF RECORD COPY

(PCT Rule 24.2(a))

To:

RESNICK, David, S.
Nixon Peabody LLP
101 Federal Street
Boston, MA 02210
ETATS-UNIS D'AMERIQUE

Date of mailing (day/month/year)	IMPORTANT NOTIFICATION
19 October 2000 (19.10.00)	
Applicant's or agent's file reference	**International application No.**
1039/48929 P	PCT/US00/24839

The applicant is hereby notified that the International Bureau has received the record copy of the international application as detailed below.

Name(s) of the applicant(s) and State(s) for which they are applicants:

CHILDREN'S MEDICAL CENTER CORPORATION (for all designated States except US)
BERNFIELD, Merton et al (for US)

International filing date	:	11 September 2000 (11.09.00)
Priority date(s) claimed	:	10 September 1999 (10.09.99)
Date of receipt of the record copy by the International Bureau	:	05 October 2000 (05.10.00)
List of designated Offices	:	

EP :AT,BE,CH,CY,DE,DK,ES,FI,FR,GB,GR,IE,IT,LU,MC,NL,PT,SE
National :AU,CA,JP,US

ATTENTION

The applicant should carefully check the data appearing in this Notification. In case of any discrepancy between these data and the indications in the international application, the applicant should immediately inform the International Bureau.

In addition, the applicant's attention is drawn to the information contained in the Annex, relating to:

- [X] time limits for entry into the national phase
- [X] confirmation of precautionary designations
- [X] requirements regarding priority documents

A copy of this Notification is being sent to the receiving Office and to the International Searching Authority.

The International Bureau of WIPO
34, ch min des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer:
V. Gross

Telephone No. (41-22) 338.83.38

INFORMATION ON TIME LIMITS FOR ENTERING THE NATIONAL PHASE

The applicant is reminded that the "national phase" must be entered before each of the designated Offices indicated in the Notification of Receipt of Record Copy (Form PCT/IB/301) by paying national fees and furnishing translations, as prescribed by the applicable national laws.

The time limit for performing these procedural acts is **20 MONTHS** from the priority date or, for those designated States which the applicant elects in a demand for international preliminary examination or in a later election, **30 MONTHS** from the priority date, provided that the election is made before the expiration of 19 months from the priority date. Some designated (or elected) Offices have fixed time limits which expire even later than 20 or 30 months from the priority date. In other Offices an extension of time or grace period, in some cases upon payment of an additional fee, is available.

In addition to these procedural acts, the applicant may also have to comply with other special requirements applicable in certain Offices. **It is the applicant's responsibility** to ensure that the necessary steps to enter the national phase are taken in a timely fashion. Most designated Offices do not issue reminders to applicants in connection with the entry into the national phase.

For detailed information about the procedural acts to be performed to enter the national phase before each designated Office, the applicable time limits and possible extensions of time or grace periods, and any other requirements, see the relevant Chapters of Volume II of the PCT Applicant's Guide. Information about the requirements for filing a demand for international preliminary examination is set out in Chapter IX of Volume I of the PCT Applicant's Guide.

GR and ES became bound by PCT Chapter II on 7 September 1996 and 6 September 1997, respectively, and may, therefore, be elected in a demand or a later election filed on or after 7 September 1996 and 6 September 1997, respectively, regardless of the filing date of the international application. (See second paragraph above.)

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

CONFIRMATION OF PRECAUTIONARY DESIGNATIONS

This notification lists only specific designations made under Rule 4.9(a) in the request. It is important to check that these designations are correct. Errors in designations can be corrected where precautionary designations have been made under Rule 4.9(b). The applicant is hereby reminded that any precautionary designations may be confirmed according to Rule 4.9(c) before the expiration of 15 months from the priority date. If it is not confirmed, it will automatically be regarded as withdrawn by the applicant. There will be no reminder and no invitation. Confirmation of a designation consists of the filing of a notice specifying the designated State concerned (with an indication of the kind of protection or treatment desired) and the payment of the designation and confirmation fees. Confirmation must reach the receiving Office within the 15-month time limit.

REQUIREMENTS REGARDING PRIORITY DOCUMENTS

For applicants who have not yet complied with the requirements regarding priority documents, the following is recalled.

Where the priority of an earlier national, regional or international application is claimed, the applicant must submit a copy of the said earlier application, certified by the authority with which it was filed ("the priority document") to the receiving Office (which will transmit it to the International Bureau) or directly to the International Bureau, before the expiration of 16 months from the priority date, provided that any such priority document may still be submitted to the International Bureau before that date of international publication of the international application, in which case that document will be considered to have been received by the International Bureau on the last day of the 16-month time limit (Rule 17.1(a)).

Where the priority document is issued by the receiving Office, the applicant may, instead of submitting the priority document, request the receiving Office to prepare and transmit the priority document to the International Bureau. Such request must be made before the expiration of the 16-month time limit and may be subjected by the receiving Office to the payment of a fee (Rule 17.1(b)).

If the priority document concerned is not submitted to the International Bureau or if the request to the receiving Office to prepare and transmit the priority document has not been made (and the corresponding fee, if any, paid) within the applicable time limit indicated under the preceding paragraphs, any designated State may disregard the priority claim, provided that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity to furnish the priority document within a time limit which is reasonable under the circumstances.

Where several priorities are claimed, the priority date to be considered for the purposes of computing the 16-month time limit is the filing date of the earliest application whose priority is claimed.

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION CONCERNING SUBMISSION OR TRANSMITTAL OF PRIORITY DOCUMENT

(PCT Administrative Instructions, Section 411)

To:

RESNICK, David, S.
Nixon Peabody LLP
101 Federal Street
Boston, MA 02210
ETATS-UNIS D'AMERIQUE

Date of mailing (day/month/year) 15 December 2000 (15.12.00)	
Applicant's or agent's file reference 1039/48929 P	**IMPORTANT NOTIFICATION**
International application No. PCT/US00/24839	International filing date (day/month/year) 11 September 2000 (11.09.00)
International publication date (day/month/year) Not yet published	Priority date (day/month/year) 10 September 1999 (10.09.99)
Applicant CHILDREN'S MEDICAL CENTER CORPORATION et al	

1. The applicant is hereby notified of the date of receipt (except where the letters "NR" appear in the right-hand column) by the International Bureau of the priority document(s) relating to the earlier application(s) indicated below. Unless otherwise indicated by an asterisk appearing next to a date of receipt, or by the letters "NR", in the right-hand column, the priority document concerned was submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b).

2. This updates and replaces any previously issued notification concerning submission or transmittal of priority documents.

3. An **asterisk(*)** appearing next to a date of receipt, in the right-hand column, denotes a priority document submitted or transmitted to the International Bureau but not in compliance with Rule 17.1(a) or (b). In such a case, **the attention of the applicant is directed** to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

4. The **letters "NR"** appearing in the right-hand column denote a priority document which was not received by the International Bureau or which the applicant did not request the receiving Office to prepare and transmit to the International Bureau, as provided by Rule 17.1(a) or (b), respectively. In such a case, **the attention of the applicant is directed** to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

Priority date	Priority application No.	Country or regional Office or PCT receiving Office	Date of receipt of priority document
10 Sept 1999 (10.09.99)	60/153,310	US	12 Dece 2000 (12.12.00)

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer

Magda BOUACHA

Telephone No. (41-22) 338.83.38

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark
Office, PCT
2011 South Clark Place Room
CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 13 July 2001 (13.07.01)	
International application No. PCT/US00/24839	**Applicant's or agent's file reference** 1039/48929 P
International filing date (day/month/year) 11 September 2000 (11.09.00)	**Priority date** (day/month/year) 10 September 1999 (10.09.99)
Applicant BERNFIELD, Merton et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

03 April 2001 (03.04.01)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Juan Cruz
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38